WHAT CAUSES MYOPIA? Complex genetics and epidemiology of a common condition

Virginie J. M. Verhoeven

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WHAT CAUSES MYOPIA?
Complex genetics and epidemiology of a common condition

HOE ONTSTAAT MYOPIE?
Complexe genetica en epidemiologie van een veelvoorkomende aandoening

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Prof. dr. G.P.M. Luyten

CONTENTS

1.1

Aims of this thesis

AIMS OF THIS THESIS

Myopia—and high myopia in particular—is more than merely an optical aberration. It is a highly prevalent eye disorder caused by elongation of the eyeball (Figure 1); in severe cases, myopia can lead to blindness. The worldwide prevalence of myopia is rising, as is the number of individuals who have become blind and/or visually impaired due to this disease. In the Netherlands today, at least one in three individuals is myopic. In recent decades, research into the causes of myopia has increased considerably. Family reports and twins studies suggest a strong influence of genetic background, and epidemiology studies have suggested that environmental factors—including near work, reading, education, and outdoor exposure—contribute to the risk of developing myopia. Although recent scientific breakthroughs have underscored the notion that myopia results from a complex interplay between nature and nurture, how these factors are interrelated and cause disease at the molecular level has remained unclear. Thus, more extensive research into the causes of myopia and refractive error is clearly needed.

In this thesis, we addressed the following questions:

Chapter 1: What is the current state of knowledge regarding the genetic epidemiology of myopia and refractive error?

Chapter 2: What are the prevalence and visual consequences of myopia and refractive errors among the general population?

Chapter 3: Which genetic risk factors are associated with the development of refractive error and endophenotypes?

Chapter 4: How do environmental factors influence the development of myopia, and are gene-environment interactions involved in this process?

Chapter 5: Can we describe functional mechanisms that play a role in the development of myopia?

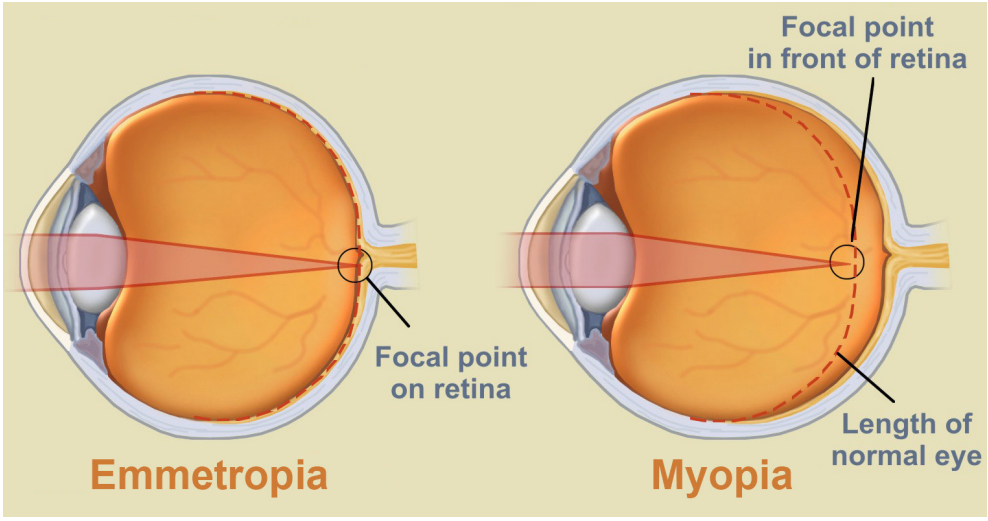

Figure 1. Normal eye without refractive error (emmetropia, left) and with myopia (right)

1.2

General Introduction: Why do eyes become myopic?

Adapted from: Caroline C.W. Klaver, Jan R. Polling, Jan W.L. Tideman, Magda A. Meester-Smoor, **Virginie J.M. Verhoeven**, *Cataract & Refractive Surgery Today Europe*, June 2014

The original publication can be found at: http://bmctoday.net/crstodayeurope/pdfs/1014CRSTEuro_Fundamentals.pdf

REFRACTIVE ERROR AND MYOPIA

Refractive errors (myopia, hyperopia, and astigmatism) are complex heterogeneous disorders of the human eye. Myopia (nearsightedness) is a common eye condition predominantly caused by an elongation of the eye's axial length. With myopia, collimated light entering the eye produces an image that is focused in front of the retina rather than on the retina. This error causes blurred vision in the distance and can usually be corrected with negative glasses, contact lenses, and/or laser refractive surgery. Unfortunately, however, this elongation of the eye can lead to structural changes in the retina and/or optic disc, particularly in patients with a high degree of myopia.

The most important determinants of refractive errors are axial length and corneal curvature[1-3]. Axial length and corneal curvature are highly correlated, and minimal changes in these parameters lead to large changes in refractive error[4,5]. Axial length is the primary determinant of refractive error and is based on a combination of anatomical factors, including anterior chamber depth, lens thickness, and vitreous chamber depth (Figure 1).

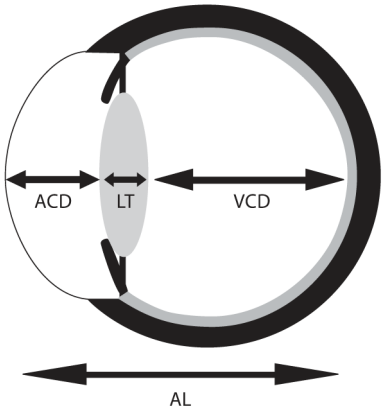

Figure 1. Anatomical fractions of axial length
Adapted from Meng et al.[2]; ACD = anterior chamber depth; LT = lens thickness; VCD = vitreous chamber depth; AL = axial length

Although axial length measurement is more objective, precise, and reproducible compared to assessment of refractive status, the latter is usually used in clinical practice to define myopia. Refractive error (measured in diopters; D) can be easily measured automatically using an auto-refractor. Refractive error is usually analyzed in terms of spherical equivalent (SE), which can be calculated using the following formula: SE = [sphere + (½ cylinder)].

Prevalence

Refractive errors are the most common eye disorders worldwide and are the leading cause of visual impairment[6,7]. Reports have shown that the prevalence of myopia is on the rise[8-10]. For example, in the United States, the prevalence of myopia increased by 145% in the past three decades, and the rate of high myopia (defined as refractive error greater than –6 D) increased by 820%[9]. In South Korea, the prevalence of myopia and high myopia increased by 334% and 891%,

respectively, in the same time period[10]. Although the same trends occurred in African and European populations[11-13], the prevalence of myopia is currently the highest among Asian populations. For example, 80% to 90% of all young adults in Singapore is myopic[14]. These dramatic figures are illustrated graphically in Figure 2.

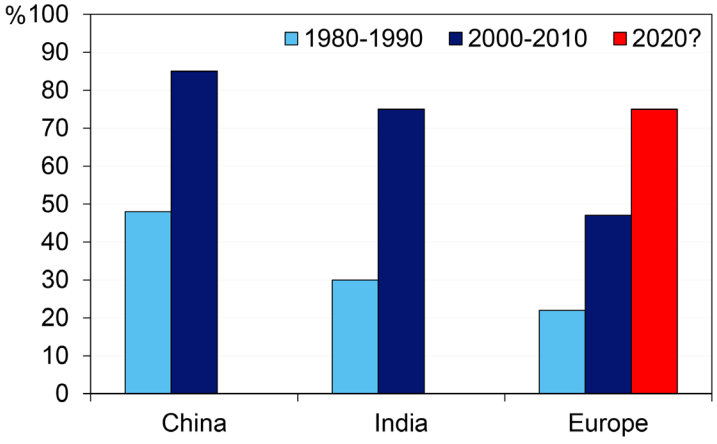

Figure 2. Increasing prevalence of myopia in Asia and Europe, with the estimated prevalence in Europe for the year 2020[14,57]

High myopia (defined as more than -6 diopters) is associated with a significant risk of developing visual complications, including myopic macular degeneration, glaucoma, and retinal detachment (Figure 3)[15-17]. However, data regarding the absolute risk of visual impairment among individuals with (high) myopia are not available.

Burden of disease

In the next ten years, an estimated 2.5 billion people worldwide will have myopia[6], more than 10% of these people (375 million) will have high myopia, and 49 million will develop severe visual impairment as a result of this condition. In addition to placing a considerable burden on the quality of life of the affected individuals and their families, this outcome can also have major financial consequences[18,19]. Given the current lack of adequate treatment modalities, the expected increase in new myopic patients—including here in the Netherlands—will create a significant burden in the coming ten years in terms of both our public health and our economy.

Course of refractive error and the onset of myopia

Children are born hyperopic and usually become emmetropic by 6-9 years of age due to a process known as emmetropization[20]. Although the cornea generally stabilizes at around six years of age[21], the power of the lens usually continues to change until age 12, and the eye's axis can continue to elongate into adulthood (i.e., until 20-25 years of age)[21,22]. The severity of adult myopia is inversely correlated with the age of onset: the onset of high myopia usually occurs in the first decade of life, whereas mild myopia often develops in the teenage years or early adulthood.

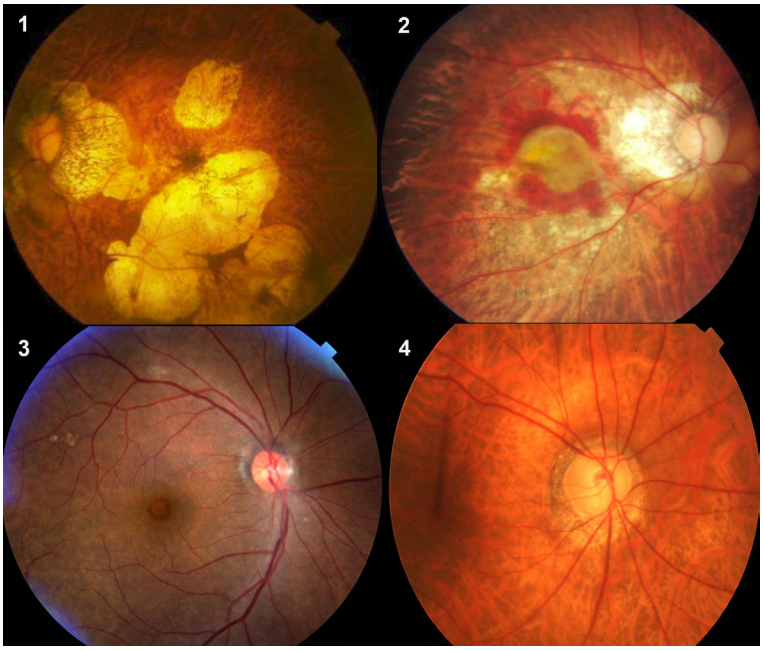

Figure 3. Examples of complications that can occur in patients with high myopia: 1. chorioretinal atrophy; 2. subretinal neovascularization; 3. macular hole; and 4. glaucoma

Current hypothesis regarding the pathogenesis of myopia

The currently accepted hypothesis regarding the pathogenesis of myopia is that excessive eye growth is induced by a visually evoked signaling cascade that originates in the retina, traverses the retinal pigment epithelium (RPE) and choroid, and terminates in the sclera, where active remodeling of the extracellular matrix (ECM) causes the eye to become elongated. However, the physical events that trigger this cascade, the cell types involved, and the biochemical drivers of this cascade are currently unknown.

RISK FACTORS FOR DEVELOPING MYOPIA

Genetic risk factors

Evidence for the heritability of refractive error and myopia generally stems from studies of familial clustering[23], high heritability values in twins[3,24], and high occurrence rates in offspring[25-27]. With respect to refractive error, the proportion of phenotypic variation that can be attributed to genetic variation is estimated at >90%[24].

Myopia is a common feature of several heritable connective tissue disorders, including Marfan syndrome (OMIM #154700), which is caused by mutations in the fibrillin-1 gene (*FBN1*), and Stickler syndrome (OMIM #108300 and #604841), which is caused by mutations in the *COL2A1* and *COL11A1* genes[28]. Associations between these loci and common myopia have been suggested only for *COL2A1*[29,30].

The search for genes that underlie the heritability of myopia was initiated by linkage studies among families and high-risk groups, yielding several loci associated with refractive phenotypes (MYP 1-18)[7,27,31,32]. Familial linkage studies have highlighted the heterogeneous genetic etiology of refractive error. Searches for additional candidate genes yielded positive associations with several genes, including genes that encode collagens (*COL2A1* and *COL1A1*[29,30]), transforming growth factors (*TGFβ1, TGFβ2,* and *TGIF1*[33-35]), hepatocyte growth factor and its receptor (*HGF* and *CMET*[36-39]), insulin-like growth factor (*IGF1*[40,41]), matrix metalloproteinases (*MMP1*, *MMP2*, *MMP3*, and *MMP9*[42,43]), and the ocular developmental gene *PAX6* [44].

Although these studies yielded some associations, a general lack of validation emerged across studies. A more powerful and successful approach is genome-wide association study (GWAS) analysis, which robustly investigates numerous single-nucleotide polymorphisms (SNPs) across the genome in large populations (Figure 4)[45].

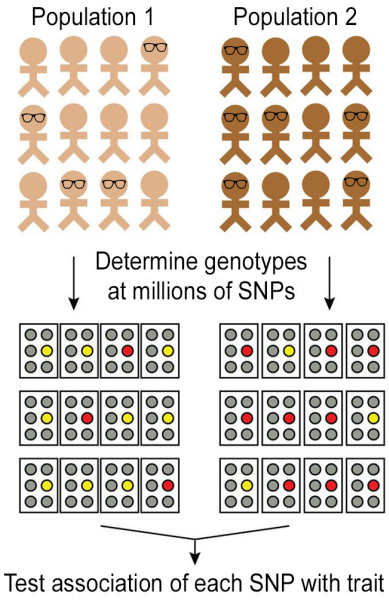

Figure 4. The principle of a genome-wide association study

Environmental risk factors

Without doubt, myopia is caused by both nature and nurture. Environmental factors are increasingly viewed as triggers for onset and progression of myopia[46,47]. In particular, education is an important risk factor; the risk of developing myopia is up to four times higher in persons with a university-level education compared to persons with only primary schooling[48]. Similar effects are observed for urban versus rural areas[49]. Two factors appear to contribute to these associations: (1) myopic children spend less time outdoors than non-myopic children, and (2) they perform more near work at an earlier age[48]. Exactly why being outdoors is so protective is unclear, but several animal experiments suggest that dopamine release in the retina triggered by the high light intensity

outdoors slow the elongation of the eyeball[50]. Why near work is detrimental is unclear as well, but animal studies on this topic suggest that near work increases hyperopic defocus in the peripheral retina, and thereby forms a trigger for eye growth[51].

Gene-environment interactions

In most diseases that have both genetic and environmental factors, these two categories of factors generally have considerable interactions[31,48,52]. To date, gene-gene and gene-environment interactions have not been studied systematically for myopia or refractive error. Because interrelationships between genes and the environment also determine a high proportion of the variance in the disease[53], a shift in focus from the current approach of a purely genetic dissection to the identification of gene-environment interactions may be challenging, but it is essential for identifying the missing links in myopia.

CURRENT THERAPEUTIC OPTIONS

The current treatment strategies for myopia are limited. Atropine, a muscarinic receptor antagonist that can be applied topically to the eye, has been the most effective in terms of inhibiting eye growth[54]. Unfortunately, atropine has unfavorable side effects, including photophobia and blurred vision while performing near work, thus reducing patient compliance[55]. Optical correction, including cornea-reshaping contact lenses, can also slow the progression of myopia; however, these approaches are generally less effective than atropine[56].

These currently available measures will not be sufficient to counteract the predicted increase in new myopes; thus, there is a clear growing need for new treatments that are both effective and tolerated.

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2.1

Prevalence of refractive error in Europe: the European Eye Epidemiology (E^3) Consortium

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ABSTRACT

Purpose

To estimate the prevalence of refractive error in adults across Europe.

Methods

Refractive data (mean spherical equivalent) collected between 1990 and 2013 from fifteen population-based cohort and cross-sectional studies of the European Eye Epidemiology (E^3) Consortium were combined in a random effects meta-analysis stratified by 5-year age intervals and gender. Participants were excluded if they were identified as having had cataract surgery, retinal detachment, refractive surgery or other factors that might influence refraction. Estimates of refractive error prevalence were obtained including the following classifications: myopia ≤-0.75 diopters (D), high myopia ≤-6D, hyperopia ≥1D and astigmatism ≥1D.

Results

Meta-analysis of refractive error was performed for 61,946 individuals from fifteen studies with median age ranging from 44-81 and minimal ethnic variation (98% European ancestry). The age-standardised prevalences (using the 2010 European Standard Population, limited to those ≥25 and <90 years old) were: myopia 30.6% (95% Confidence Interval (CI) 30.4-30.9), high myopia 2.7% (95% CI 2.69-2.73), hyperopia 25.2% (95% CI 25.0-25.4) and astigmatism 23.9% (95% CI 23.7-24.1). Age-specific estimates revealed a high prevalence of myopia in younger participants (47.2% (CI 41.8-52.5) in 25-29 years-olds).

Conclusions

Refractive error affects just over a half of European adults. The greatest burden of refractive error is due to myopia, with high prevalence rates in young adults. Using the 2010 European population estimates, we estimate there are 227.2 million people with myopia across Europe.

INTRODUCTION

Refractive error occurs when there is failure of the eye to correctly focus rays of light from an object onto the retinal plane. The resultant image perceived by the individual is blurred and refractive correction is required in order to see clearly. Refractive error can be divided into myopia ('short or near-sightedness'), hyperopia ('long or far-sightedness') and astigmatism. In myopia, light is focussed to a point anterior to the retina as a result of excessive refraction at the cornea or lens, or, more commonly, an increased length of the eye ('axial myopia'). In hyperopia, the reverse occurs with an image forming posterior to the retinal plane as a result of either inadequate refraction or a short axial length. In astigmatism, the refractive power of the eye is uneven across different meridians.

Refractive error requires detection and treatment in the form of glasses, contact lenses or, more recently, refractive surgery. These clinical services are readily available in most European countries, although they come with significant financial implications to both national health care systems and to individuals[1]. However, uncorrected refractive errors are still responsible for up to 42% of the cases of visual impairment worldwide[2], and remain prevalent even in high income countries[3-6]. Uncorrected refractive error in both low and high-income countries has significant economic implications in terms of potential lost productivity[7].

The magnitude of refractive error in developed countries within individuals of European descent has been estimated by the Eye Diseases Prevalence Research Group, ten years ago, and the US National Health and Nutrition Examination Survey (NHANES) data[3,8]. However, the estimate of refractive error burden in Europe was based on a single cohort[9]. The European Eye Epidemiology (E^3) consortium is a collaborative initiative between thirty-three cohort studies across Europe, to share and meta-analyse epidemiological data on eye disease in adults. The aim of the current study was to provide more current and precise estimates of the prevalence of refractive error across Europe.

MATERIAL AND METHODS

Studies and participants

To date, E^3 has data from thirty-three studies with a range of ophthalmic data on approximately 124,000 individuals from population-based and case-control studies. This study drew on the fifteen E^3 population-based cohort and cross-sectional studies that collected refractive error data (n=68,350). As described in Table 1, participants included in this meta-analysis were largely from Northern and Western Europe, mainly of middle to late age, and refractive error measurements were performed between 1990 and 2013. Three studies recruited participants nationally and the remaining twelve recruited from a local population. Further detail on individual study design and sampling method is provided in Table 1; broadly, the majority of study samples were obtained by identification of potential participants (within defined age bands and/or regions) using local registries, with some studies using random sampling (n=3). All studies adhered to the tenets of the Declaration of Helsinki, and relevant local ethical committee approvals with specific study consent were obtained.

Inclusion and exclusion criteria

Studies in the E^3 consortium were eligible for inclusion in this analysis if they were population-based, and data on refraction, together with age at measurement and year of birth, were available. Study participants were excluded if they were identified as having had cataract surgery, retinal detachment, refractive surgery or other factors that might influence refraction (eg. keratoconus), at the discretion of each study's analysis team.

Demographic and outcome variables

All included studies measured non-cycloplegic refraction (i.e. no dilating drops were used) using the technique of subjective refraction, autorefraction or a combination of focimetry (measuring an individuals glasses) or autorefraction followed by subjective refraction (Table 1). Participant's spherical equivalent (SE) was considered as the mean SE of the two eyes calculated using the standard formula (SE = sphere + (cylinder/2)). Refractive error was categorized using the following definitions: myopia ≤ -0.75 diopters (D), low myopia ≤ -0.75 to > -3 D, moderate myopia ≤ -3 D to > -6 D, high myopia ≤ -6 D, hyperopia ≥ 1 D, high hyperopia ≥ 3 D and astigmatism ≥ 1 D. Definitions of myopia vary in the literature; the cut-off of -0.75 D was chosen as unaided visual acuity at this level approximates 0.3 LogMAR (Logarithm of the Minimum Angle of Resolution)[10], a commonly used driving standard, and this has been used in recent international meta-analyses of the genetic epidemiology of refractive error and myopia[11].

Differences in age (in five year age bands from ≥15 years to ≥90 years), gender (male/female) and geographical European region were examined. Geographical variations in the prevalence of myopia were investigated by dividing countries in three areas (Northern, Western and Southern Europe) according to the United Nations Geoscheme[12]. Information on ethnicity, when available, was recorded using a modified classification system based on genetic ancestry[13].

Table 1. Description of the 15 European Eye Epidemiology Consortium studies included in this meta-analysis of refractive error

Study	Data collection period	Study design	Sampling method	Total with refraction	Refraction method	Exclusions (cataract surgery)	Total included	Median age, years (range)	Gender, % female	Ethnicity, % European (% Unknown)	Crude myopia prevalence, %	Crude hyperopia prevalence, %
Northern Europe												
1958 British Birth Cohort, UK	2002-2003	Population-based birth cohort (N)	Systematic (birth week in 1958)	2502	Autorefraction	7 (0)	2495	44 (44-46)	51.7	98.0 (9.2)	48.7	8.8
EPIC-Norfolk, UK	2004-2011	Population-based cross-sectional study (L)	Systematic	8508	Autorefraction	1110 (971)	7444	67 (48-92)	54.5	99.7 (0)	23.0	39.4
Tromsø Eye Study, Norway	2007-2008	Population-based cohort (L)	Systematic/ Random	6565	Autorefraction	773 (700)	5792	61 (38-87)	55.9	NA (100)	19.4	33.7
TwinsUK, UK	1998-2010	National twin cohort (N)	Volunteer	6245	Autorefraction	161 (61)	6095	55 (16-85)	91.2	98.2 (23.9)	31.4	26.0
Southern Europe												
Thessaloniki Eye Study, Greece	1999-2005	Cross-sectional population-based study (L)	Systematic	2259	Subjective	316 (303)	1952	69 (60-94)	44.7	100 (0)	14.2	39.4
Western Europe												
ALIENOR, France	2006-2008	Population-based cohort L)	Systematic	951	Autorefraction	333 (318)	618	79 (73-93)	56.6	NA (100)	16.7	53.6
ERF, Netherlands	2002-2005	Family-based cross-sectional study (L)	Systematic (genetically isolated population)	2708	Subjective	46 (45)	2662	49 (14-87)	55.1	100 (0)	21.2	27.4
Gutenberg Health Study, Germany	2007-2012	Population-based cohort (L)	Systematic	14679	Autorefraction	610 (610)	14069	54 (35-74)	49.4	NA (100)	31.9	23.9
KORA, Germany	2004-2005	Population-based cohort (L)	Systematic	3078	Autorefraction	706 (177)	2372	55 (35-84)	50.4	100 (0)	36.1	24.0
Montrachet, France	2009-2013	Population-based cohort (L)	Systematic	1143	Autorefraction	584 (562)	576	81 (76-92)	57.5	NA (100)	19.1	51.1
Rotterdam Study I, Netherlands	1990-1993	Population-based cohort (L)	Systematic	6748	Subjective	182 (172)	6566	68 (55-106)	59.3	98.5 (2.0)	16.4	52.3
Rotterdam Study II, Netherlands	2000-2002	Population-based cohort (L)	Systematic	2689	Subjective	110 (110)	2579	62 (55-99)	54.8	87.8 (0.1)	21.9	45.7
Rotterdam Study III, Netherlands	2005-2008	Population-based cohort (L)	Systematic	3624	Subjective	94 (74)	3530	56 (46-97)	56.3	NA (100)	32.5	28.8
POLA, France	1995-1997	Population-based cohort (L)	Systematic	2464	Autorefraction	157 (128)	2315	70 (60-93)	55.8	NA (100)	16.2	53.0
Mixed												
EUREYE: Norway, UK, France, Italy, Greece & Estonia	2000-2002	Population based cross-sectional survey in seven cities(L)	Random sample from population registers in each city	4187	Autorefraction or focimetry with subjective refraction	1305 (517)	2882	72 (65-95)	56.7	NA (100)	15.6	59.2

Myopia ≤ -0.75 diopters (D), hyperopia ≥ 1D, N = national, L = local

Statistical analysis

Study specific summary data were obtained. A random effects meta-analysis was performed for spherical equivalent and repeated for refractive classifications overall and stratified by age. This enabled calculation of pooled estimates of refractive error prevalence, with studies weighted by sample size and between-study variance and a summary estimate standard error calculated from the inverse sum of the adjusted weights. A random effects model was chosen over a fixed effects model, to allow for heterogeneity in study design characteristics.

Age-standardised prevalences were calculated using the following steps: firstly, age-specific prevalences were estimated using random-effect meta-analyses. Secondly, an age-standardisation with adjustments to age-specific estimates according to the European Standard Population 2010 was performed[14]. This enabled refractive error prevalence estimates that are representative for the European population, with appropriate weighting to the age demographic distribution of Europe.

Subsequent random effects meta-analyses were performed with stratification by age and gender, and subsequently age and geographical region, with differences between groups evaluated using ANOVA tests.

Statistical analysis was performed using Stata version 13.1 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP). Graphical outputs were obtained using either Stata or ggplot2[15] in R (R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org).

RESULTS

Fifteen studies contributed a total of 61,946 individuals after exclusions (Figure 1). The median age of the included populations ranged from 44 to 78 years old (Table 1). There was a slight female predominance in the combined study (57.6% females). Data on ethnicity was only available for 50% of participants, and in these there was minimal ethnic diversity (98% European ancestry), so no further analysis of ethnicity was carried out.

The distribution of refractive error displayed a leptokurtotic distribution (Figure 2), with a median spherical equivalent of 0.56 D (range -25.13 to 22.19). The distribution was asymmetric with a greater frequency of individuals with a negative refractive error.

Given there were only 314 participants aged 15-24 years and 156 greater than 90 years of age, subsequent analyses are limited to those aged ≥25 and <90 years (n=61,476). The overall myopia prevalence in our meta-analysis was 24.2% (95% Confidence Interval (CI) CI 19.9-28.5), with a European age-standardised myopia prevalence of 30.6% (95% CI 30.4-30.9) (Table 2). Myopia was most common in younger participants (peaking at 47.2% (95% CI 41.8-52.5) in those aged 25-29 years), almost double the prevalence of those of middle and older age (27.5% (95% CI 23.5-31.5) in those aged 55-59 years) (Figure 3A). Point estimates of myopia prevalence in those aged 15-19 years were 27.4% (95% CI 17.0-37.8), increasing to 34.2% (95% CI 27.9-40.6) in those aged 20-24 years. All degrees of myopia followed a similar pattern of higher prevalence in

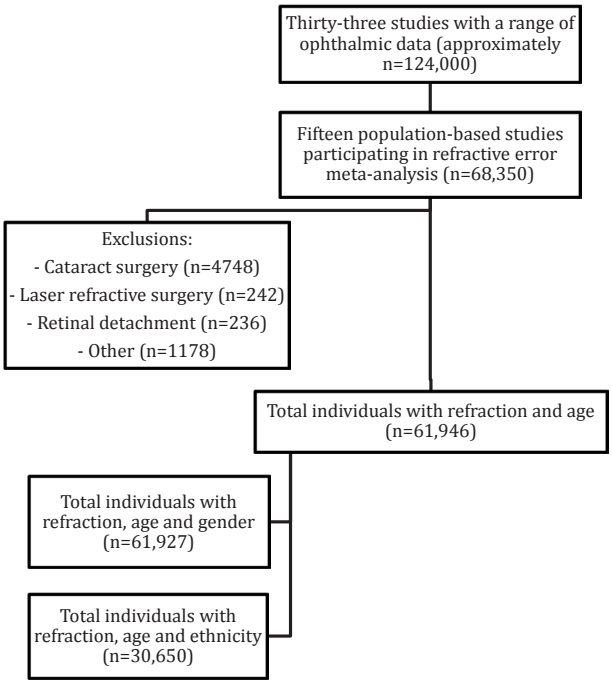

Figure 1. Flow chart of refractive error meta-analysis within E³

the younger cohorts, lower prevalence in the middle aged and more elderly participants, and an increase in the very eldest participants, albeit with wide confidence intervals, most likely related to cataract development. Age-standardised prevalence of high myopia across all age groups was 2.71% (95% CI 2.69-2.73), with 3-5% of young to middle-aged individuals affected and 1-2% of older individuals (Figure 3B).

Overall prevalence of hyperopia was 34.7% (95% CI 27.9-41.6), with an age-standardised prevalence of 25.2% (95% CI 25.0-25.4). There was less hyperopia in young participants (6.4% (95% CI 3.8 - 9.0) in those aged 25-29 years), compared to those in middle to older age (31.2% (95% CI 27.5-34.9) in those aged 55-59 years) although hyperopia rates declined after 75 years of age. The prevalence of high hyperopia followed a similar pattern, affecting 1-3% of younger and 10-13% of older individuals (Figure 3C). Across all ages, the prevalence of astigmatism was 27.3% (95% CI 22.6-32.1) with an age-standardised estimate of 23.9% (95% CI 23.7-24.1). The prevalence of astigmatism remained fairly stable at 15-25% in young and middle-aged participants (17.0% (95% CI 15.1-18.8) in those aged 45 to 49 years). However, in participants over 65 years of age, astigmatism became more common (51.1% (95% CI 40.4-61.8) in those aged 80-84 years) (Figure 3D).

Age- and gender- specific analyses for myopia, hyperopia and astigmatism are reported in Table 3. There were no significant differences in myopia prevalence between men and women across age strata. However, overall there was a significantly higher prevalence of astigmatism in men ($p=0.001$), with a mean difference of 3.8% across all ages, and a significantly higher prevalence

Table 2. Prevalence of myopia, hyperopia and astigmatism stratified by age

Age	n	Myopia, % (95% Confidence Intervals)				Hyperopia, % (95% Confidence Intervals)		Astigmatism, % (95% Confidence Intervals)
		All Myopia ≤ -0.75 D (n=15,845)	Low Myopia ≤ -0.75 to > -3 D (n=10,034)	Moderate Myopia ≤ -3 to > -6 D (n=4,383)	High Myopia ≤ -6 D (n=1,445)	All Hyperopia ≥ +1 D (n=21,201)	High Hyperopia ≥ +3 D (n=4,494)	All Astigmatism ≥ 1 D (n=15,496)
25-29	339	47.2 (41.8 - 52.5)	26.5 (21.8 - 31.2)	14.1 (5.1 - 23.2)	5.3 (2.9 - 7.7)	6.4 (3.8 - 9.0)	1.1 (0.0 - 2.2)	16.2 (12.3 - 20.1)
30-34	469	38.3 (22.6 - 53.9)	25.5 (16.7 - 34.2)	9.4 (4.2 - 14.6)	3.2 (1.5 - 4.9)	5.5 (3.4 - 7.5)	1.8 (-1.1 - 4.6)	18.2 (14.3 - 22.0)
35-39	2354	40.1 (29.2 - 51.0)	25.8 (15.5 - 36.0)	10.0 (7.9 - 12.1)	3.7 (1.3 - 6.1)	5.8 (3.0 - 8.6)	1.4 (0.5 - 2.3)	16.2 (14.5 - 17.9)
40-44	5552	40.2 (32.0 - 48.5)	27.5 (19.7 - 35.3)	9.6 (7.0 - 12.3)	3.3 (1.8 - 4.8)	7.9 (6.3 - 9.5)	2.2 (1.6 - 2.7)	15.7 (13.2 - 18.1)
45-49	4108	37.1 (29.4 - 44.7)	25.1 (18.8 - 31.4)	9.0 (6.5 - 11.4)	2.9 (1.8 - 4.0)	10.3 (7.5 - 13.2)	2.4 (1.7 - 3.1)	17.0 (15.1 - 18.8)
50-54	5684	33.6 (29.6 - 37.6)	20.9 (18.6 - 23.2)	9.8 (8.0 - 11.6)	2.7 (1.4 - 4.0)	18.0 (15.6 - 20.4)	3.3 (2.6 - 3.9)	20.1 (16.3 - 23.8)
55-59	8294	27.5 (23.5 - 31.5)	16.6 (14.2 - 18.9)	8.3 (6.6 - 9.9)	2.5 (1.9 - 3.1)	31.2 (27.5 - 34.9)	5.7 (4.6 - 6.8)	22.5 (18.2 - 26.9)
60-64	10594	21.4 (17.5 - 25.2)	13.0 (10.9 - 15.2)	6.0 (4.5 - 7.4)	2.0 (1.4 - 2.7)	31.2 (27.5 - 34.9)	7.5 (6.0 - 9.0)	25.2 (20.3 - 30.0)
65-69	9445	15.9 (13.7 - 18.1)	9.8 (8.4 - 11.2)	4.7 (3.7 - 5.7)	1.4 (1.1 - 1.6)	50.2 (46.1 - 54.3)	9.7 (8.2 - 11.1)	28.0 (22.0 - 34.0)
70-74	7674	13.9 (11.9 - 15.9)	9.3 (7.8 - 10.9)	3.4 (2.8 - 4.0)	1.0 (0.6 - 1.5)	54.3 (50.4 - 58.1)	12.8 (9.9 - 15.7)	33.8 (26.6 - 41.1)
75-79	4211	15.9 (13.4 - 18.4)	10.2 (8.5 - 11.8)	3.9 (2.9 - 5.0)	1.5 (1.0 - 1.9)	56.3 (52.1 - 60.4)	12.8 (9.9 - 15.7)	44.3 (33.6 - 55.0)
80-84	2069	17.8 (15.2 - 20.3)	11.5 (10.1 - 12.9)	3.8 (2.7 - 4.9)	1.5 (1.0 - 2.1)	52.8 (47.9 - 57.7)	12.0 (9.7 - 14.3)	51.1 (40.4 - 61.8)
85-89	683	17.9 (14.0 - 21.8)	12.4 (9.0 - 15.8)	3.4 (2.0 - 4.8)	1.4 (0.4 - 2.3)	49.2 (42.5 - 55.9)	13.4 (8.4 - 18.5)	54.9 (42.9 - 66.8)
Age standardised prevalence (n=61,476)		30.60 (30.36 - 30.85)	19.50 (19.35 - 19.65)	8.08 (8.01 - 8.14)	2.71 (2.69 - 2.73)	25.23 (25.03 - 25.43)	5.37 (5.33 - 5.41)	23.86 (23.67 - 24.05)

D, diopters

Table 3. Prevalence of myopia, hyperopia and astigmatism stratified by age and gender

Age	n		Myopia, ≤ -0.75 D (95% Confidence Intervals)		Hyperopia, ≥ +1 D (95% Confidence Intervals)		Astigmatism, ≥ 1D (95% Confidence Intervals)	
	Women	Men	Women	Men	Women	Men	Women	Men
25-29	278	59	47.9 (40.0 - 55.8)	40.2 (22.7 - 57.8)	6.1 (6.1 - 6.2)	11.2 (-1.5 - 23.8)	14.9 (12.0 - 17.7)	19.6 (15.9 - 23.3)
30-34	307	160	40.3 (32.6 - 47.9)	41.7 (10.7 - 72.7)	4.3 (0.0 - 8.6)	5.2 (2.9 -7.5)	15.0 (6.7 - 23.2)	22.4 (19.4 - 25.5)
35-39	1352	1004	40.2 (30.5 - 49.8)	40.9 (26.2 - 55.5)	5.6 (3.0 - 8.1)	6.5 (3.8 - 9.2)	14.3 (11.7 - 16.9)	14.9 (11.1 - 18.7)
40-44	2989	2561	39.8 (31.4 - 48.2)	42.0 (33.4 - 50.6)	7.9 (6.6 - 9.1)	8.4 (7.0 - 9.8)	14.8 (12.5 - 17.1)	18.7 (16.3 - 21.0)
45-49	2258	1849	37.1 (29.8 - 44.3)	37.1 (25.8 - 48.3)	11.4 (9.1 - 13.8)	8.3 (6.6 - 9.9)	16.3 (14.4 - 18.2)	17.1 (16.0 - 18.2)
50-54	3369	2315	34.1 (30.0 - 38.3)	33.0 (27.7 - 38.3)	19.1 (17.6 - 20.7)	17.3 (15.1 - 19.5)	19.9 (15.8 - 23.9)	20.9 (16.9 - 25.0)
55-59	5086	3206	25.8 (21.6 - 30.1)	29.8 (23.7 - 35.9)	32.6 (29.8 - 35.5)	30.6 (25.9 - 35.2)	20.9 (17.8 - 23.9)	25.5 (18.8 - 32.1)
60-64	6364	4226	19.1 (15.9 - 22.3)	20.7 (17.0 - 24.4)	43.7 (38.8 - 48.6)	36.1 (31.9 - 40.2)	21.8 (18.5 - 25.1)	24.4 (19.4 - 29.4)
65-69	5207	4237	14.5 (12.0 - 17.1)	16.6 (14.2 - 18.9)	52.3 (48.2 - 56.4)	48.4 (44.7 - 52.1)	25.9 (20.9 - 30.9)	32.1 (26.3 - 37.8)
70-74	4110	3562	14.2 (12.2 - 16.2)	14.3 (12.3 - 16.3)	55.8 (52.2 - 59.4)	55.0 (51.5 - 58.5)	31.4 (25.5 - 37.3)	38.3 (31.5 - 45.1)
75-79	2290	1920	14.3 (11.5 - 17.0)	17.7 (14.9 - 20.5)	58.0 (55.3 - 60.6)	52.3 (48.0 - 56.7)	39.9 (35.1 - 57.2)	46.2 (35.1 - 57.2)
80-84	1158	911	15.8 (12.8 - 18.7)	18.7 (16.2 - 21.3)	57.6 (53.0 - 62.2)	47.8 (42.6 - 53.0)	50.0 (37.6 - 62.4)	55.8 (46.3 - 65.3)
85-89	419	264	19.6 (13.2 - 25.9)	16.1 (11.7 - 20.5)	50.7 (43.5 - 57.9)	45.0 (37.9 - 52.0)	55.5 (40.3 - 70.6)	53.8 (40.2 - 67.3)
p diff between groups (ANOVA)			0.603		0.042		0.001	

D, diopters

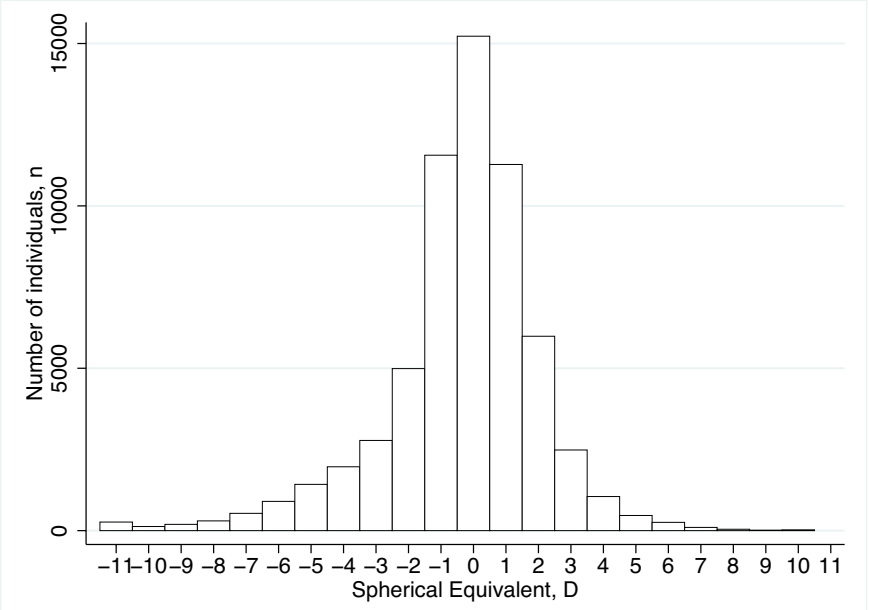

Figure 2. Distribution of refractive error
D, diopters

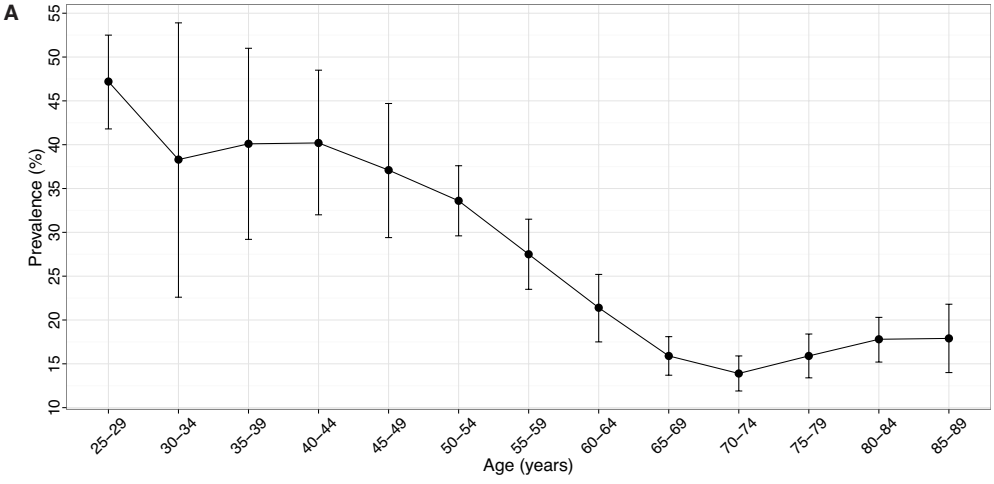

Figure 3A. Prevalence of myopia (SE ≤ -0.75D) according to age, with 95% confidence intervals
D, diopters

of hyperopia in women (p=0.04) with a mean difference of 2.5% across all ages.

Differences in the myopia prevalence between different European regions, according to the UN European Geoscheme, were examined. Only one cohort contributed to the Southern European division (Thessaloniki Eye Study, Greece), with participants all over the age of 60 years, thus the majority of the studies were in Northern and Western regions. The prevalence of myopia did not differ between Northern and Western countries and followed a similar pattern across all age

groups. The single Southern participant cohort appeared to have a higher level of myopia in its older participants when compared to Northern and Western countries, however there were large confidence intervals for these estimates (80-84 year-old myopia prevalence in North 13.6% (95% CI 9.3-18.0), West 18.0% (95% CI 16.1-21.1) and South 29.1% (95% CI 19.1-39.1). Overall there were no significant differences across age strata between the three regions of Europe studied (p=0.70).

B

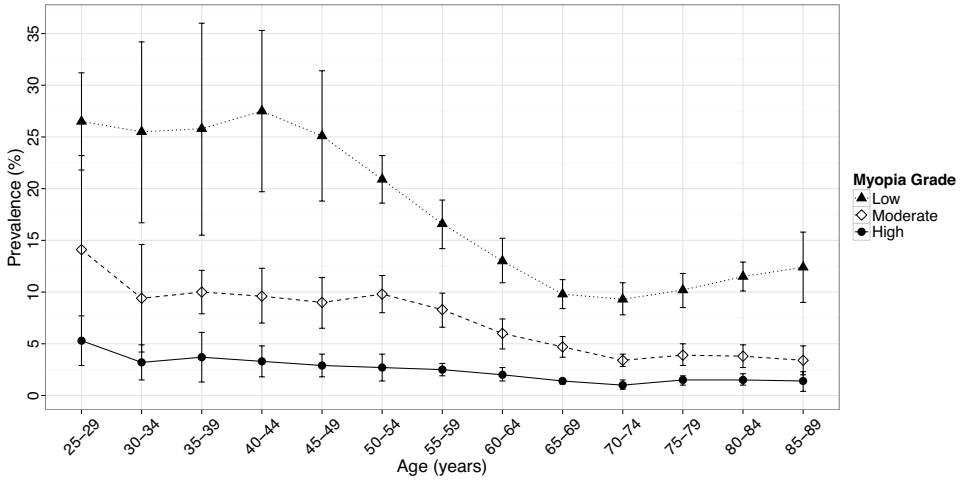

Figure 3B. Prevalence of myopia (low myopia SE ≤ -0.75 to > -3D, moderate myopia SE ≤ -3 to > -6D, high myopia SE ≤ -6D) according to age, with 95% confidence intervals
D, diopters

C

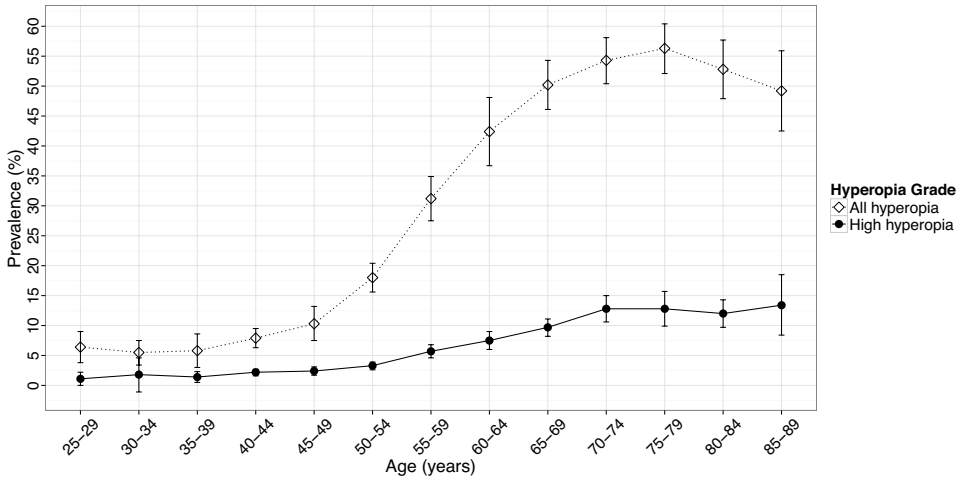

Figure 3C. Prevalence of hyperopia (all hyperopia SE ≥ 1D, high hyperopia SE ≥ 3 D), according to age, with 95% confidence intervals
D, diopters

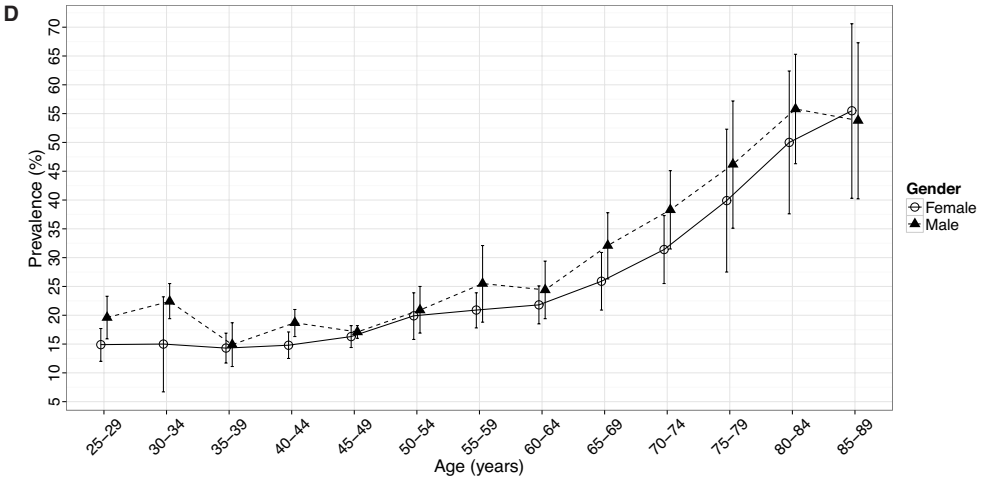

Figure 3D. Prevalence of astigmatism (≥ 1 D) according to age for males and females with 95% confidence intervals
D, diopters

DISCUSSION

Meta-analysed data from fifteen population-based adult cohort and cross-sectional studies across Europe indicated age-standardised prevalence of 30.6% for myopia, 25.2% for hyperopia and 23.9% for astigmatism. This meta-analysis usefully incorporates data from across Europe and is not limited to a particular place or age group. The most significant burden of refractive error within Europe was from myopia.

A clear trend of higher levels of myopia in younger individuals was identified, with a rising prevalence during late teens and 20s reflecting the known natural history of the condition[16]. The peak prevalence of myopia was identified in the 25-29 years age group (47.2% (95% 41.8-52.5). In older individuals, the prevalence of myopia was lower, for example 15.9% (95% CI 13.7-18.1) in those aged 65-69 years old. This may reflect the rising prevalence of myopia in younger generations, or the known hyperopic shift in aging[17,18]. In our aged 75 or over participants, there was an increase in myopia prevalence. While we aimed to exclude those having undergone cataract surgery (and participants with documented cataract in some studies), the rise in myopia likely reflects the development of nuclear cataract, which is known to be associated with a myopic shift as a result of increasing lens power[19]. However, this age-related change in refraction may also occur irrespective of visible lens opacity; in the Beaver Dam Study, a ten-year longitudinal myopic shift (-0.19D, 95% CI -0.32 to -0.06, p<0.001) was observed in those over 70 years old, even after adjusting for nuclear sclerosis grading[17]. We did not confirm the observation of previous studies of higher myopia prevalence in women[20].

In comparison to previous estimates, the overall burden of myopia in our population appears similar but slightly greater to that of other studies. The 2004 Eye Diseases Prevalence Research Group estimated myopia prevalence at 26.6%, 25.4% and 16.4% for European, North American

and Australian sub-analyses respectively[8]. This study included the Beaver Dam Eye Study[21], the Baltimore Eye Survey[22], the Blue Mountains Eye Study[23], the Melbourne Visual Impairment Project[24] and the Rotterdam Study I[25], which was also included in this meta-analysis. In their youngest cohort (40-49 years), 36.8% of white men and 46.3% of white women were myopic, similar to our estimates of 42.0% and 39.8% in 40-44 year-olds, albeit with no gender difference. The US 1999-2004 National Health and Nutrition Examination Survey (NHANES) examined refractive error variation by age in three ethnicities; the prevalence of myopia in non-Hispanic white participants 20-39 years of age was 35.1% in men and 42.3% in women, whilst the prevalence in those ≥ 60 years was 23.1% in men and 18.6% in women[20]. These prevalence rates are again very similar to that found in our data, although we did not find higher levels of myopia in young females. Both comparative estimates are based on a definition of myopia ≤ -1D, and are therefore not directly comparable to our study definition of myopia ≤ -0.75D, an issue often encountered in refractive error epidemiology where there is a lack of consensus on definitions of refractive error. The adult prevalence of myopia in South-east Asia is of much greater magnitude than that seen in studies of European ancestry[26-29], with remarkably high levels of myopia seen in young individuals[30,31]. The number of participants in our meta-analysis of Asian origin was very low, precluding meaningful reporting of these estimates.

High myopia prevalence was relatively low in Europe, with an age-standardised estimate of 2.7% (95% CI 2.69-2.73). The highest prevalence was observed in younger participants, albeit with wider confidence intervals due to smaller sample size (Table 2). Prevalence in older participants was low, potentially reflective of generational changes, or perhaps exclusion due to the earlier need for cataract surgery in high myopes compared to other refractive groups[32]. Our greatest high myopia prevalence of 5.9% (95% CI 1.3-10.5) in 15-19 year-olds remains much lower than that seen in, for example, urban China where up to 14% of 17 year-olds are highly myopic[33]. In non-Hispanic White individuals in the NHANES 1999-2004 data, high myopia appeared slightly more common than in our data; for example in those aged 20-29 years-old “severe” myopia was identified in 7.4%, compared to 2.8% and 5.3% in those aged 20-24 and 25-29 respectively in this European study. However the NHANES definition of severe myopia (≤ -5D) again differs slightly from our definition of high myopia (≤ -6D).

Using the same definition of high hyperopia (≥ 3D), our study appeared to have less hyperopia than the Eye Diseases Research Group[8]; for example in 70-74 year-olds 21.3% of white women and 16.9% of white men were highly hyperopic compared to just 12.8% in our European data, which may again reflect a generational or cohort effect.

Astigmatism rates were fairly constant (15-25%) across cross-sectional age categories, but were higher after the age of 65. This finding has been observed in other studies, together with a shift from with-the-rule to against-the-rule astigmatism[20,23,29]. Across all age groups, we identified higher astigmatism prevalence in men, particularly evident in middle to later ages (for example 39.5% in women and 46.2% in men aged 70-74). This observation was similar in the older participants of the NHANES 1999-2004 study, where in participants over the age of 60 years the astigmatism prevalence in women was 46.1% and in men 54.9%[20].

The major strength of our study is the large sample size contributing to the prevalence estimates, providing a unique opportunity to estimate the burden of refractive error in middle and older aged individuals across Europe. This is beneficial for planning of clinical services and raises awareness, for both clinicians and economists, of the future potential issues of rising myopia levels and associated visual impairment[34]. Refractions were all non-cycloplegic, which is common practice for population-based adult ophthalmic epidemiological studies, thus making this study comparable to previous research[35,36].

Despite age and gender stratification, significant heterogeneity between studies remained in the meta-analysis. There are inherent differences in the included studies in terms of study design, refraction technique and cohort sampling, together with between country differences in levels of urbanisation, economy, education and climate which may influence refractive error. We were unable to stratify by these factors in this meta-analysis as person-specific data was not available for all studies. This study was mainly comprised of middle and older aged individuals, therefore our estimates of refractive error prevalence carry greater confidence for these ages since they are based on more precise estimates with narrow 95% confidence intervals. The majority of the studies in this meta-analysis originate from Northern and Western European countries, and therefore our estimates of refractive error are more representative of these European countries. Although our sample includes either national or locally recruited population-based studies, like all epidemiological studies there may be a bias of participants volunteering for an eye examination being more 'health conscious'. We suspect this would have little effect on the prevalence of refractive error, and if anything result a slight underestimation of the prevalence. Finally, refractions were performed over a twenty-year period and, therefore our estimates of prevalence may be subject to error given temporal trends in refractive error prevalence. However, refractions were performed between 2000 and 2010 in thirteen out of the fifteen studies, reducing this variability.

In conclusion, this study estimates refractive error affects just over a half of European adults. Myopia represented the greatest burden, with an estimated 227.2 million people across Europe affected (using the 2010 European population estimates)[37]. Based on study prevalence estimates of high myopia, this also suggests there are 20.1 million people across Europe who are at higher risk of the associated sight threatening complications, such as retinal detachment, that this degree of myopia infers[34].

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2.2

Visual consequences of refractive errors in the general population

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Supplementary files and figures can be found at: http://www.aaojournal.org/article/S0161-6420(14)00641-1/abstract

ABSTRACT

Objective

To study the frequency and causes of visual impairment in relation to refractive error.

Design

Population-based cohort study

Participants

A total of 6,597 participants from Rotterdam Study I (baseline and 4 follow-up examinations) and of 2,579 participants from Rotterdam Study II (baseline and 2 follow-up examinations), all aged 55+ years, were included.

Methods

Participants underwent an extensive ophthalmic examination including best-corrected visual acuity and objective refraction, fundus photography, visual field perimetry, and OCT imaging of macula and optic disc. We calculated cumulative risks and odds ratios of visual impairment for various refractive error categories, determined causes by using all screening information as well as medical records. Main Outcome Measures**:** Unilateral and bilateral low vision (WHO criteria: VA <0.3 and VA ≥0.05; US criteria: VA <0.5 and VA ≥0.1) and blindness (WHO criteria: VA <0.05; US criteria: VA<0.1).

Results

Cumulative risks of visual impairment ranged from virtually 0 in all refractive error categories at age 55 to 9.5% (standard error (se) 0.01) for emmetropia, 15.3% (se 0.06) for high hyperopia to 33.7% (se 0.08) for high myopia, at age 85. The major causes of visual impairment in highly hyperopic persons were age-related macular degeneration (AMD), cataract, and combined causes (each 25%); in highly myopic persons the major cause was myopic macular degeneration (38.9%). The major causes of visual impairment for the other refractive error categories were AMD and cataract. Compared to emmetropes, high myopes had a significantly increased risk of visual impairment; those with ≤-6 D & ≥-10 D had a risk of OR 3.4 (95% CI 1.4-8.2) of visual impairment; those with <-10 D had OR 22.0 (95% CI 9.2-52.6).

Conclusion

Of all refractive errors, high myopia has the most severe visual consequences. Irreversible macular pathology is the most common cause of visual impairment in this group.

INTRODUCTION

Refractive errors - both myopia and hyperopia - are very common human eye disorders and leading causes of visual impairment worldwide.[1-3] Myopia is characterized by an elongation of the eye, and is accompanied by structural changes of the retina and choroid.[4] These changes can lead to potentially blinding complications such as myopic macular degeneration, open-angle glaucoma and retinal detachment.[5,6] Although all myopic eyes are at risk for complications[4,7,8], highly myopic eyes, i.e., -6 diopters (D) or worse, are particularly at risk to develop functional blindness at a relatively young age. Hyperopia (farsightedness), by contrast, is a condition in which the eye is shortened. For this refractive error category, the risks of visual impairment are less well studied, but it is known that persons with hyperopia have a higher risk of amblyopia, strabismus and closed-angle glaucoma.[9] An association with age-related macular degeneration (AMD) has also been described.[10]

Although numerous studies have addressed population frequencies of low vision and blindness none have focused on visual loss as a function of the full spectrum of refractive errors. In addition, frequency of causes of blindness and low vision specified per refractive error category have not been described until now. Given the current rise in prevalence of this trait[11-13], this information can be useful for clinicians, patients, and researchers, and will increase awareness of the visual consequences of refractive errors.

In this study, we investigated the frequency and causes of blindness and low vision stratified for various refractive error categories in 2 independent cohorts of the population-based prospective Rotterdam Study.

MATERIAL AND METHODS

Study population

The rationale and design of the Rotterdam Study have been described in detail elsewhere.[14] In brief, this prospective population-based follow-up study focuses on chronic ophthalmologic, neurologic, cardiovascular, and locomotor diseases in middle aged and elderly participants living in Ommoord, a city district of Rotterdam, the Netherlands. Baseline data for the ophthalmic part were collected between 1991 and 2002 and follow-up examinations were performed at 2-4 years (Figure 1). A total of 99% of study participants were from European descent. For this analysis, we included 9,176 participants from two independent cohorts of the Rotterdam Study. The first is Rotterdam Study I (RS-I): 6,597 participants aged 55 years and older. Baseline examinations took place between 1990 and 1993, and four follow-up examinations were performed in 1993-1995, 1997-1999, 2002-2004, and 2009-2011 (Figure 1). The second cohort is Rotterdam Study II (RS-II), which included 2,579 participants aged 55 years and older. Baseline examinations took place in between 2000 and 2002, and two follow-up examinations were performed in 2004-2005 and 2011-2012 (Figure 1). Persons with bilateral pseudophakia or aphakia at baseline with no knowledge of prior refractive error were excluded (n = 278). From these two cohorts, 9,176 participants with data on refractive error and visual acuity at baseline were eligible for the current analysis. The Medical Ethics Committee of the Erasmus University had approved the study protocols, and participants had given a written informed consent in accordance with the Declaration of Helsinki.

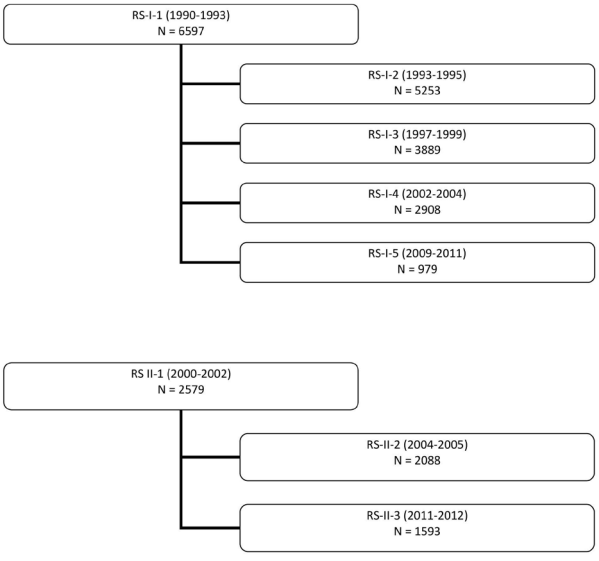


Figure 1. Participation and ophthalmological measurement from each examination interval of the Rotterdam Study
RS, Rotterdam Study

Ophthalmic data collection

All patients underwent an extensive ophthalmological examination. Visual acuity was measured using the Lighthouse Distance Visual Acuity Test, a modified Early Treatment Diabetic Retinopathy Study chart.[15] To evaluate the best-corrected visual acuity (BCVA), refraction was initially obtained after objective autorefraction (Topcon RM-A2000, Topcon Optical Company, Tokyo, Japan), and then subjectively adjusted. Screening of visual fields was performed using a modified 76-point supra-threshold perimetry test (Humprey Visual Field Analyzer, Zeiss, Oberkochen, Germany); visual field defects were confirmed by Goldmann perimetry. After pupil dilation, optic nerve head and macular area imaging was performed using simultaneous stereoscopic photography (Topcon TRC-SS2, Topcon optical Company, Tokyo, Japan), followed by a 35° film fundus camera (Topcon TRV-50VT, Topcon Optical Company, Tokyo, Japan). During the last examination rounds, RSI-4, RSI-5 and RSII-2 respectively, a Topcon digital 35° colour fundus camera (Topcon TRC 50EX with a Sony DXC-950P digital camera; 0.44 megapixel) was used.

Low vision and blindness were classified according to the WHO criteria[16] and US criteria:

Low vision: WHO: VA <0.3 and ≥0.05; US: VA <0.5 and ≥0.1
Blindness: WHO: VA <0.05; US: VA<0.1

For participants with bilateral blindness and low vision, three clinical investigators (C.C.W.K, V.J.M.V., and K.T.W.) reached consensus on the final determination of the cause of visual impairment after reviewing all screening information, fundus transparencies, and medical information provided by ophthalmologists.

Statistical analysis

Mean spherical equivalent (SE) was calculated according to the standard formula (SE=spherical value + ½*cylinder). When data from only one eye were available, the SE of this eye was used. Mean SE was categorized into high myopia (≤ -6 diopters (D)), moderate myopia (>-6D & ≤-3D), low myopia (<-3D & ≤-0.75D), emmetropia (>-0.75D & <0.75D), low hyperopia (≥0.75D & <3D), medium hyperopia (≥ 3D & <6D), and high hyperopia (≥ 6D), using previously defined criteria.[17] High myopia and high hyperopia were further classified as high myopia <-10 D and ≤-6 D & ≥-10 D and high hyperopia >10 D and ≥6 & ≤10 D. Visual acuity at last visit was categorized into normal vision, low vision, and blindness according to WHO and US criteria as defined above. For bilateral visual impairment, BCVA was used. Unilateral visual impairment was defined as visual impairment in only one eye.

We calculated the number of cases with bilateral and unilateral blindness and low vision as a percentage of the total number of all cases with blindness and low vision at the endpoint of the study per refractive error category.

Cumulative risks of bilateral visual impairment were estimated per refractive error category using Kaplan Meier product limit analysis. We assigned the age at diagnosis of blindness or low vision as the mean between the examination at which this endpoint was first observed and the previous examination. For participants who did not develop the endpoint, we used age at last examination for censoring. Participants who died or were lost to follow-up were counted at the time of the last

examination. All participants aged 85+ years were censored at age 85 years to maintain unbiased estimates. Cumulative risks per refractive error category were compared with the log-rank test of equality (Mantel-Cox) using emmetropia as the reference group.

Causes of bilateral blindness and low vision (according to the WHO criteria) were categorized, and frequencies of causes were calculated per refractive error category. We calculated mean age at diagnosis of bilateral visual impairment per refractive error category, and calculated mean spherical equivalent per refractive error category, stratified by normal vision, low vision and blindness. Statistical differences at nominal *P*-value <0.05 between refractive error categories for age at diagnosis and between visual acuity categories for mean SE were calculated using Student's T test. The risk of blindness and low vision (reference normal vision) for persons with various refractive error categories (reference emmetropia) was assessed using logistic regression analysis with blindness and low vision as a combined outcome, correcting for age and sex. We used SPSS version 20.0.0 (SPSS Inc.) for all analyses.

RESULTS

General characteristics of the 9,176 study participants are presented in Table 1. At baseline, we identified 98 prevalent cases (1.1%) with bilateral low vision and 29 cases (0.3%) with bilateral blindness (WHO criteria). After a mean follow-up time of 9.6±6.1 years, respectively 62 and 26 persons developed incident bilateral low vision and blindness. Subjects in RS-I were generally younger (mean age at inclusion 69.0 versus 64.1 years) and were less myopic (mean SE 0.84 vs. 0.47 D) than those in RS-II, due to a cohort effect described in our previous work.[17] The characteristics of all cases who had received a diagnosis of bilateral low vision or blindness by the end of the study can be found in Table 2 (WHO criteria) and Table 3 (US criteria; available at http://aaojournal.org).

The distribution of bilateral and unilateral blindness and low vision (WHO criteria) per refractive error category is shown in Figure 2. The high myopia group showed the highest percentage of bilateral blindness (9.6%) and low vision (25.0%). Persons from the high hyperopia group had the highest proportion of unilateral blind eyes (39.1%).

Kaplan Meier curves showing cumulative risk of visual impairment for high myopia, emmetropia and high hyperopia appear in Figure 3. Cumulative risks ranged from virtually 0 in all refractive error categories at age 55 to 9.5% (standard error (se) 0.01) for emmetropia, 15.3% (se 0.06) for high hyperopia to 33.7% (se 0.08) for high myopia, at age 85. Risks for high myopia started to increase gradually before age 60; for high hyperopia between 60 and 70 years of age, whereas emmetropia showed a more steady increase in risk from the age of 70. Cumulative risks for persons with low to moderate myopia and hyperopia were not significantly different from persons with emmetropia (*P* 0.09; *P* 0.78). Kaplan Meier curves for US criteria can be found in Figure 4 (available at http://aaojournal.org). Cumulative risks ranged from virtually 0 in all refractive error categories at age 55 to 28.9% (standard error (se) 0.03) for emmetropia, 41.5% (se 0.08) for high hyperopia to 59.2% (se 0.08) for high myopia, at age 85.

Table 1. Characteristics of the study population

	Rotterdam Study I	Rotterdam Study II	Total
N at baseline	6597	2579	9176
Follow-up time, mean ± SD (yrs)	9.8 ± 6.0	8.9 ± 2.9	9.6 ± 6.1
Baseline age, mean ± SD (yrs)	69.0 ± 9.0	64.1 ± 7.4	67.6 ± 8.8
Sex, % men	41.0	45.0	42.0
Visual acuity at last measurement - WHO criteria			
Bilaterally visually impaired subjects, %	2.2	0.5	1.7
Bilaterally blind subjects, %	0.8	0.1	0.6
Unilaterally visually impaired subjects, %	6.1	3.8	5.5
Unilaterally blind subjects, %	3.4	2.1	3.0
Visual acuity at last measurement - US criteria			
Bilaterally visually impaired subjects, %	6.6	1.8	5.2
Bilaterally blind subjects, %	1.1	0.1	0.8
Unilaterally visually impaired subjects, %	12.5	4.8	10.3
Unilaterally blind subjects, %	3.4	2.2	3.1
Refractive error			
Spherical equivalent, mean ± SD (D)	0.84 ± 2.54	0.47 ± 2.49	0.74 ± 2.53
High myopia ≤-6D, %	1.8	1.8	1.8
Medium myopia >-6D & ≤-3D, %	5.2	7.3	5.8
Low myopia -3D & ≤-0.75D, %	9.5	12.8	10.4
Emmetropia >-0.75D & <0.75D, %	25.4	26.9	25.8
Low hyperopia ≥0.75D & <3D, %	44.4	41.1	43.4
Medium hyperopia ≥3D & <6D, %	12.3	9.2	11.4
High hyperopia ≥6D, %	1.5	1.0	1.3

SD, standard deviation; D, diopters; WHO, World Health Organization

Table 2. Characteristics of all cases with bilateral blindness, low vision and normal vision at the end point of the study (WHO criteria)

	Bilaterally blind subjects N = 55	Bilaterally visually impaired subjects N = 160	Subjects with bilateral visual acuity ≥ 0.3 N = 8961
Age of onset, mean ± SD (yrs)	78.1 ± 11.3	79.7 ± 10.1	NA
Range age of onset	55.4; 96.3	56.4; 106.2	NA
Sex, % men	31.0	53.0	51.0
Spherical equivalent, mean ± SD (D)	-0.05 ± 5.78	0.09 ± 4.03	0.75 ± 2.45
Range spherical equivalent	-19.13; 12.25	-15.31; 8.50	-19.13; 15.13
High myopia ≤-6D, %	9.1	8.1	1.7
Moderate myopia >-6D & ≤-3D, %	5.5	7.5	5.7
Low myopia -3D & ≤-0.75D, %	10.9	10.6	10.4
Emmetropia >-0.75D & <0.75D, %	16.4	19.4	26.0
Low hyperopia ≥0.75D & <3D, %	38.2	38.1	43.6
Moderate hyperopia ≥3D & <6D, %	12.7	13.8	11.4
High hyperopia ≥6D, %	7.3	2.5	1.3

SD, standard deviation; D, diopters; WHO,World Health Organization; RS, Rotterdam Study; NA, not applicable

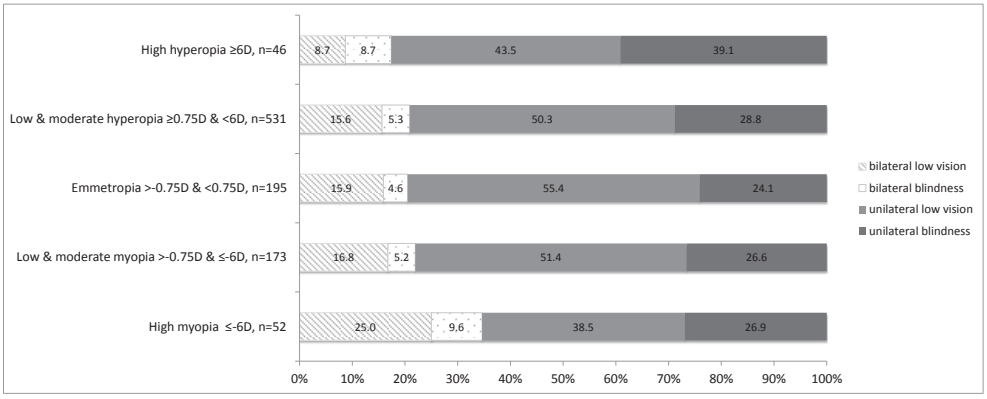


Figure 2. Distribution of bilateral and unilateral blindness and low vision (WHO criteria) per refractive error category

The number of cases with bilateral and unilateral blindness and low vision is shown as a percentage of the total number of prevalent and incident cases with blindness and low vision per refractive error category. For data of visual impairment as a percentage of the entire population, see Table 1.
WHO, World Health Organization

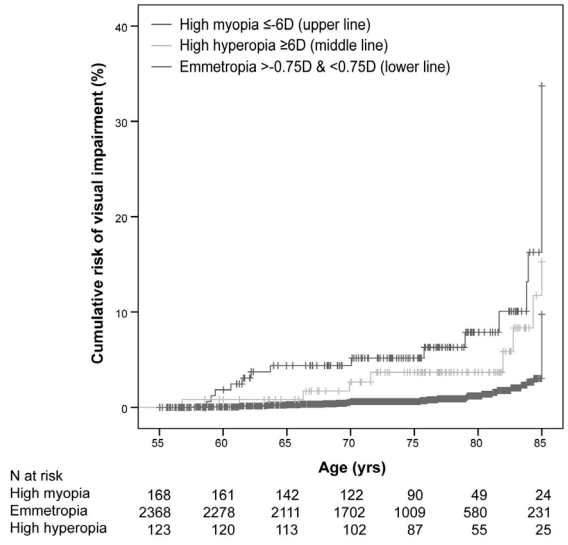


Figure 3. Cumulative risk of bilateral visual impairment (WHO criteria) stratified for high myopia, emmetropia and high hyperopia

The X-axis represents the age at diagnosis for all cases with blindness or low vision at the end point of the study and age at last examination for non-cases; the Y-axis represents the cumulative risk for persons with visual impairment. The number of persons at risk at each decade per refractive error category is presented below.

The causes of bilateral visual impairment according to WHO criteria are provided in Figure 5. For persons with emmetropia, low to moderate myopia, and low to moderate hyperopia, AMD was the major cause of visual impairment. The most important cause of visual impairment in high

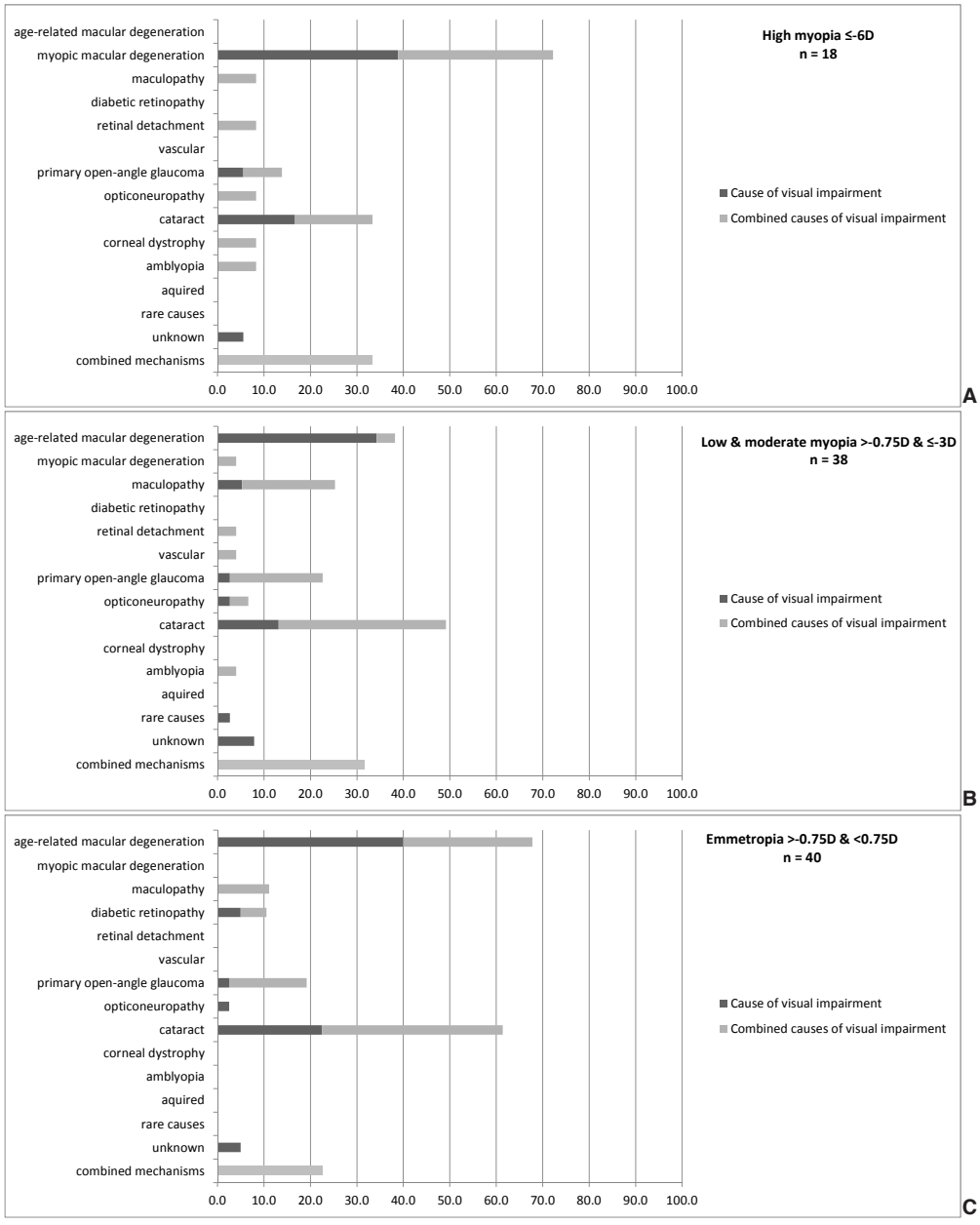
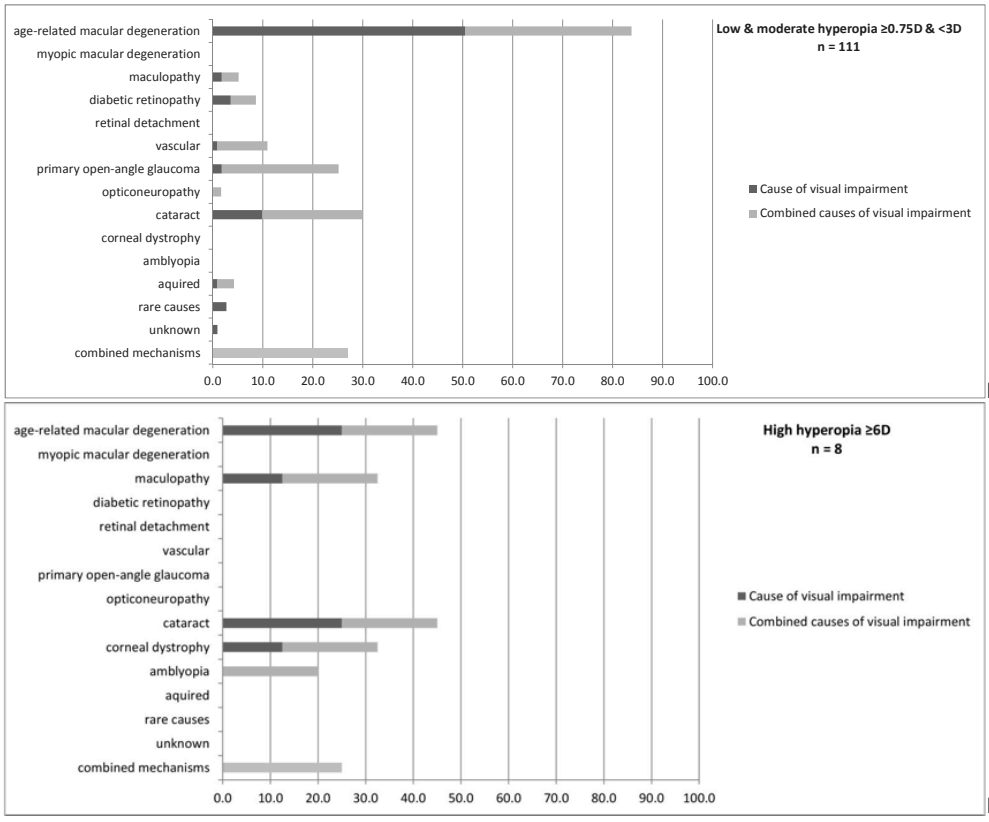


Figure 5. **Causes of bilateral low vision and blindness (WHO criteria) stratified by refractive error category**

The X-axis represents the percentage of visual impairment explained by the different causes mentioned on the Y-axis stratified by subjects with high myopia (Figure 5A), low & moderate myopia (Figure 5B), emmetropia (Figure 5C), low & moderate hyperopia (Figure 5D) and high hyperopia (Figure 5E).
D, diopters; NA, not applicable; WHO, World Health Organization



myopic persons was myopic macular degeneration (38.9%), followed by combined mechanisms (33.3, including myopic macular degeneration, cataract, and maculopathy) and cataract (16.7%). In highly hyperopic persons, the major causes of visual impairment were AMD (25%), cataract (25.0%), and combined causes (25%, including amblyopia, corneal dystrophy, cataract, maculopathy, age-related macular degeneration).

The age at diagnosis of visual impairment for persons with high myopia (75.4±13.7 yrs) and high hyperopia (75.4±10.0 yrs) was slightly, albeit non significantly, lower than for persons with emmetropia (80.3±11.0 yrs; P =0.152; and P = 0.250, respectively).

Boxplots of the SE distribution among visually impaired participants with high myopia and high hyperopia are provided in Figure 6. Among the high myopes, persons with bilateral blindness (SE=-15.25 ± 5.23 D; P = 0.034) and low vision (SE=-10.91 ± 2.57 D, P = 0.0036) had a significantly lower SE (i.e. more myopia)than persons with normal vision (SE=-8.25 ± 2.59 D). In the other refractive error groups, no statistical SE differences were found between the visual acuity categories (data not shown). The risk of blindness or low vision for high myopes versus emmetropes was OR 3.4 (95% CI 1.4-8.2, P<0.001) for those with SE ≤-6 D & ≥-10 D, and OR 22.0 (95% CI 9.2-52.6, P 0.01) for those with SE <-10 D (Figure 7).

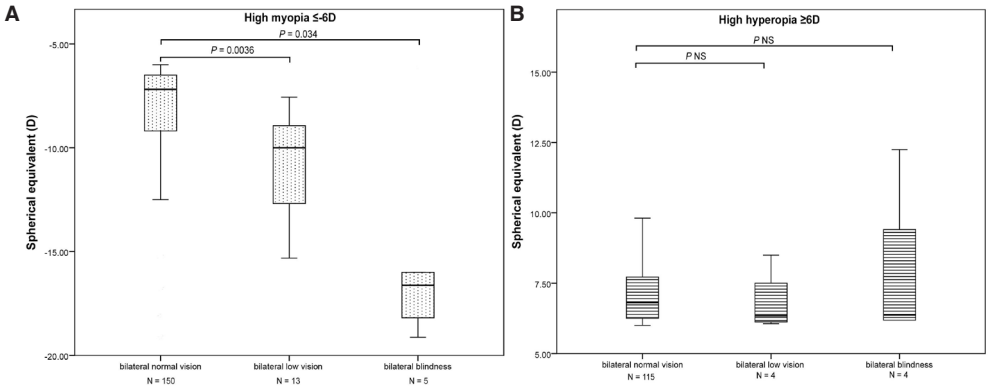


Figure 6. Distribution of spherical equivalent in relation to bilateral visual impairment (WHO criteria) in participants with high myopia (Figure 6A) and high hyperopia (Figure 6B).
Boxplots for the distribution of spherical equivalent stratified by bilateral blindness, bilateral low vision and normal vision (based on WHO criteria) for all subjects with high myopia SE ≤-6D (Figure 6A) and high hyperopia ≥ 6 D (Figure 6B).
D, diopters; WHO, World Health Organization; NS, not significant

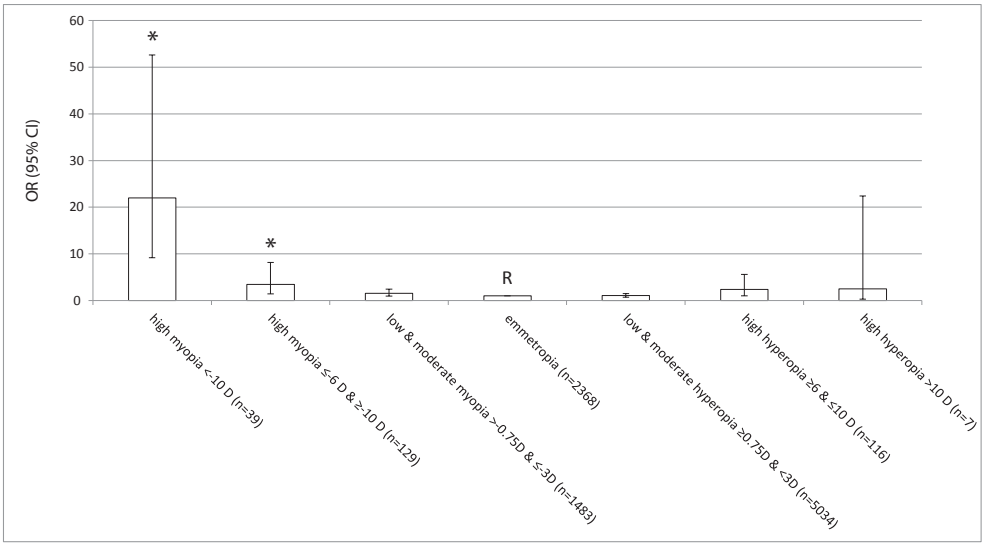


Figure 7. Risk of bilateral blindness and low vision (WHO criteria) for high myopes
Odds ratios with 95% confidence intervals for blindness and low vision (reference normal vision) for persons with high myopia with SE ≤-6 D & ≥-10 D or SE <-10 D (reference emmetropia) are shown.
CI, Confidence Interval; D, Diopters; OR, Odds Ratio; R, reference; WHO, World Health Organization; *, statistically significant OR ($P < 0.05$) compared to the reference group

DISCUSSION

In this population-based longitudinal study, we found that persons with high myopia (SE≤-6D) and high hyperopia (SE≥6D) are at a considerable risk of visual impairment. Blindness or low vision occurred in one third of high myopes, mainly caused by myopic macular degeneration. The blind and visually impaired persons within this group had a higher degree of myopia than the ones with normal vision; the risk of visual impairment was 22x increased for those with refractive errors of -10 D or more when compared to emmetropes, but also 6x higher than those with refractive errors between -6 and -10 D. The onset of visual impairment appeared to occur at a younger age; cumulative risks of visual impairment rose at least 10 years earlier for high myopia (before the age of 60) than for emmetropia (from the age of 70). For high hyperopia, we found that 15% of the persons were visually impaired. Causes of visual impairment for this refractive error showed more variation, and included cataract, AMD, and combined mechanisms.

This is the first report on refractive error specific risks and causes of blindness and low vision. Strengths of this study are the investigation of the full spectrum of refractive errors, the large sample size, and the lengthy follow-up time. In addition, our ophthalmic examination was extensive, which enabled an accurate determination of the cause of visual impairment. Our study also had limitations. Despite the large sample size, subgroup numbers were relatively small, jeopardizing precision of the risk estimates. Also, we focused on causes of visual impairment in persons with bilateral low vision, and did not study those with unilateral visual impairment. Therefore, we may have missed refractive error specific causes of visual impairment that are more likely to occur unilaterally, such as rhegmatogenous retinal detachment[18], and closed-angle glaucoma[19] in (high) myopes and amblyopia in (high) hyperopes. Lastly, selective non-participation of disabled persons may have caused an underestimation of the frequencies of blindness and visual impairment.

Our findings are in line with results from previous studies that showed a highly increased risk for high myopes (SE≤-6D).[8,20] Except for one person with moderate myopia, all persons with myopic macular degeneration were highly myopic. Those with extreme refractive error values of ≥ -10 D had the highest risk of visual impairment. We could not confirm the previously described mildly increased risk of visual impairment for persons with low to moderate myopia.[8,20]

Myopia is a growing public health problem since the prevalence is rapidly increasing, particularly in East Asia.[11-13] With time, this trend is predicted to occur in other regions as well, and the increase in myopia and high myopia prevalence will result in a higher frequency of complications. Atropine eyedrops can currently be used as a therapy in children to slow the progression of myopia and decrease the final adult value of myopic refractive error.[21] Our data underscore the objective of this therapy, because realisation of a lower refractive error will lower the risk of visual impairment later in life.[22]

It was previously shown that clinically significant pathological changes can be noted in highly myopic patients who are middle-aged or even younger.[23,24] Our mean age at diagnosis of visual impairment is likely to be overestimated, since we included persons over age 55 years with visual impairment at baseline; baseline age was 69 years for RS-I and 64.1 years for RS-II. We did not

have information on the actual age of onset of visual impairment occurring before this age.

The frequency of visual impairment in the high hyperopia group was relatively high. The number of cases with blindness or low vision in this group was very small (n = 8). Also, the proportion of high hyperopes in our older study sample was quite large, so these data are not necessarily applicable to the general population. Previous research has mainly focused on high myopia rather than on high hyperopia, but our results at least show that high hyperopia should be subject to further studies as well. Cataract was an important cause of visual impairment in all refractive error categories. This may be an overestimation of the current situation, since the majority of the data had been collected in the 1990's, and since then cataract surgery has become a more easily accessible and safer procedure. Several studies showed an increased incidence of nuclear cataract and subcapsular posterior cataract in high myopes.[25] We considered whether the exclusion of pseudophakic and aphakic persons might have introduced a selection bias and an underestimation of the risks of visual impairment in high myopic persons in our study. This does not seem to be the case, since only 2 out of 287 excluded participants (0.7%) with pseudophakia or aphakia were blind or visually impaired due to myopic macular degeneration diagnosed on the fundus photograph.

In summary, our data indicate that risks and causes of visual impairment vary with refractive error. The risks for high myopes are by far the highest with more than 1 in 3 persons with high myopia developing bilateral blindness or low vision. This large health risk requires public awareness and a focus to initiate strategies to reduce this burden in those at risk of myopia.

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2.3

Axial length and visual function in high myopia

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*These authors contributed equally to this work.
**The MYST Consortium includes all ophthalmologists who have referred high myopic cases to the MYST study.

Manuscript submitted.

ABSTRACT

Objective

To study the relationship between axial length and visual impairment in high myopia.

Design

Three population-based cohort studies (Rotterdam Study I, II, and III), one family-based study (Erasmus Rucphen Family Study, ERF), and one high-myopia case-control study (MYopia STudy, MYST).

Participants

A total of 6271 participants were included (RS-I: N = 662 (55+ years of age); RS-II: N = 1074 (55+ years); RS-III: N = 1148 (45+ years); ERF: N = 2353 (25+ years); and MYST: N = 671 cases and 363 controls, 25+ years)).

Methods

Participants received an extensive ophthalmic examination, including best-corrected visual acuity (VA), auto-refraction (spherical equivalent; SE), and measurements of axial length (AL) and corneal curvature (CC). Analyses were based on data from right eyes. The frequency of visual impairment was calculated as a function of AL (in mm), stratified by age. Cumulative risk of visual impairment (VI) per AL category (reference AL: 23-24 mm) was assessed using Kaplan-Meier product-limit analysis, .

Main Outcome Measures

Definition of high myopia: SE ≤ -6 diopters. Definition of low vision , according to the WHO criteria, VA <0.3 and VA ≥0.05; blindness VA <0.05.

Results

Mean AL in RS-I, RS-II, RS-III and ERF combined was 23.38 mm (SD: 1.15); in MYST, mean AL was 27.36 mm (SD: 1.62) in cases and 23.49 mm (SD: 0.76) in controls. The correlation of AL between right and left eyes was high (population-based R^2 = 0.906; case control R^2 = 0.932) . The frequency of VI in our total study population was 1.5%. In subjects with high myopia the frequency of VI was 5.1%, which increased with age (*P* trend <0.0001). The risk of VI was low (1.8%) in all high myopes at age 40, irrespective of AL; by age 80, however, the cumulative risk of VI was 7% for AL 23-24 mm, 30.5% for AL 26-28 mm, 44.7% for AL 28-30 mm, and 90.5% for AL ≥30 mm. Mean age of onset of visual impairment was strongly related with AL, and varied from 45 years in AL >28 mm to 58 years in AL 26-28 mm.

Conclusions

The risk of VI in high myopes is highly correlated with AL, and reaches extremely high figures in AL ≥30 mm. In high myopes, each incremental increase in AL worsens the long-term visual prognosis.

INTRODUCTION

Myopia is an extremely common form of refractive error and the major cause of visual impairment worldwide[1,2]. Myopia results from excessive growth in axial length (AL). AL is the primary determinant of refractive error and is based on a combination of anatomical factors, including anterior chamber depth, lens thickness, and vitreous chamber depth[3-5]. Although AL measurement is more objective, precise, and reproducible compared to assessments of refractive error, the latter is routinely measured in clinical practice and most commonly used to define myopia.

High myopia) is defined as a refractive error of -6 diopters (D) or worse. The AL in high myopia mostly exceeds 26 mm. High myopia occurs in 3-20% of the general population, and is currently one of the leading causes of legal blindness in developed countries due to myopic macular degeneration, retinal detachment, or glaucoma[1,6,7]. Strikingly, evidence suggests that the global prevalence of myopia and high myopia is increasing rapidly[8,9]. This alarming increase, combined with the sight-threatening complications, represents a significant medical and socio-economic burden[10].

The relationship between myopia and pathology has been addressed in several studies. These studies found that only few eyes with mild-to-moderate myopia have pathological ocular signs, whereas many eyes with high myopia develop pathologic complications[11-15]. A higher AL increases the risk of retinal lesions and myopic retinopathy, but how it relates to visual outcome is unknown. Indeed, to the best of our knowledge, no study has examined the relationship between AL and visual impairment. For the current analysis, we combined data obtained from the population-based Rotterdam I, II, and III studies, the Erasmus Rucphen Family Study, with data from the high myopia case-control study (MYST) to study cumulative risk of visual impairment as a function of axial length.

PATIENTS AND METHODS

Study population

This study included data from a total of 6271 subjects from the population-based Rotterdam Study I-III (RS-I, RS-II, and RS-III), the family-based Erasmus Rucphen Family Study (ERF), and the high-myopia case-control MYopia Study (MYST), all of which were conducted in the Netherlands. All subjects with available data on best-corrected visual acuity (BCVA), axial length, and refractive error were included in our analysis.

The Rotterdam Study is a prospective population-based cohort study of middle-aged and elderly subjects (45+ years of age) living in Ommoord, a suburb of Rotterdam, the Netherlands. The rationale and study design of this study have been described elsewhere[16]. In brief, the Rotterdam Study consists of three independent cohorts (RS-I (55+ years of age), RS-II (55+ years of age), and RS-III (45+ years of age)); this study examined cardiovascular, endocrine, neurological, respiratory, and ophthalmic outcomes. Baseline examinations—including BCVA and refractive error measurements—were performed from 1990 to 1993 (RS-I), 2000 to 2002 (RS-II), and 2006 to 2008 (RS-III). Axial length was measured in a subset of RS-III at baseline and in a subset of the studies during follow-up examinations (RS-I: 2009-2011, RS-II: 2011-2012, RS-III: 2011-2012). In total, 2284 subjects were eligible for our analysis (RS-I, N = 662; RS-II, N = 1074, RS-III, N = 1148).

The Erasmus Rucphen Family (ERF) study is a family-based study of a genetically isolated population living in the south-west of the Netherlands. This study included more than 3000 living descendants of 20 couples who lived in the region of Rucphen (the participants were 18-86 years of age at the time of the study). The subjects were examined from 2002 through 2005. The rationale and design of this study have been described elsewhere[17,18]. Only subjects ≥25 years of age were included in our study sample. In total, 2353 subjects were eligible for the analysis.

The MYopia STudy (MYST) is a high-myopia case-control study. High-myopic cases and emmetropic controls were recruited by eye care providers, opticians, and optometrists; by ophthalmologists from university hospitals (primarily from the Erasmus Medical Center, Leiden University Medical Center, and Nijmegen University Medical Center) and community hospitals (primarily from the Eye Hospital Rotterdam, the Focus Clinic Rotterdam, and the Amphia Hospital Breda); by public media outlets (www.myopiestudie.nl); and by door-to-door flyer distribution. High-myopic cases were defined as having refractive error ≤–6 D, and emmetropic controls were defined as having refractive error ≥-1.5 D and ≤1.5 D. All participants were ≥25 years of age. The subjects were examined from 2010 through 2012. In total, 1057 participants (690 cases and 367 controls) were included in this study. Of these participants, 1034 (671 cases and 363 controls) were eligible for our analysis.

Measurements in all studies were collected after receiving approval from the medical ethics committee of the Erasmus University Medical Center, and all participants provided written informed consent in accordance with the Declaration of Helsinki.

Ophthalmic examination

Participants in the RS and ERF studies received an extensive ophthalmological examination as described previously[16]. This examination included a non-cycloplegic measurement of refractive error using a Topcon RM-A2000 auto-refractor (Topcon Optical Company, Tokyo, Japan). After additional subjective refraction, BCVA was measured using the Lighthouse Distance Visual Acuity Test, a modified version of Early Treatment Diabetic Retinopathy Study (ETDRS) chart[19]. AL was measured using a Lenstar LS900 (Laméris Ootech, Haag-Streit, UK; in the RS-I, RS-II, and RS-III cohorts) or an A-scan ultrasound device (Pacscan, Sonomed Escalon, Germany; in the ERF and RS-III cohorts). Measurements of AL were introduced after the initiation phase of the RS studies; measurements of AL were available in only 25%.

The MYST study protocol included a complete ophthalmological examination, a questionnaire, and peripheral blood sampling for genotyping. A non-cycloplegic measurement of refractive error and keratometry was performed for both eyes using a Topcon RM-A2000 autorefractor (Topcon Optical Company). BCVA with objective refraction was measured using standardized ETDRS protocols[19]. Intraocular pressure was measured using Goldmann applanation tonometry. AL, corneal thickness, anterior chamber depth, and lens thickness were measured using a Lenstar LS900 (Laméris Ootech). For subjects with AL >30 mm, we used an A-scan ultrasound device (Pacscan, Sonomed Escalon) to measure AL, anterior chamber depth, and lens thickness. After dilating the pupils with tropicamide/phenylephrine 0.5/5%, we performed indirect ophthalmoscopy to quantify myopic degeneration of the retina (including the peripheral retina)[20]. Stereoscopic digital colour photographs (35°) were taken of the lens, the optic nerve head, and the macular area using a Topcon digital fundus camera (Topcon TRC 50EX; 0.44 megapixel, Topcon Optical Company) or a Sony DXC-950P digital camera (Sony Corporation, Minato, Japan). We also performed fundus autofluorescence, infrared, and red-free measurements of the macular area of each eye (Heidelberg HRA-2, Heidelberg Engineering, Heidelberg, Germany). Optic discs were imaged using confocal laser scanning tomography (Heidelberg Retina Tomograph HRT II, Heidelberg Engineering). Optical coherence tomography was performed using Topcon 1000, 2000 (SD) and Topcon-DRI (SS) (Topcon Optical Company), and scans were made of both the optic nerve and the macula. The questionnaire included questions regarding the subject's complete ocular and medical history, family history of myopia, education level, and near-work and outside activities during young childhood, adolescence, and adulthood.

In all studies, visual impairment was defined as either low vision (VA <0.3 and VA ≥0.05) or blindness (VA <0.05) according to the WHO criteria[21].

Statistical analysis

All analyses were performed on the subjects' right eyes. Spherical equivalent (SE) was calculated using the following standard formula: SE = sphere + (½ cylinder). For our analysis of SE and AL, we excluded subjects with a history of cataract surgery or refractive surgery with no prior knowledge of refractive error. For participants with both A-scan ultrasound as well as Lenstar measurements of AL, the Lenstar measurement was used. AL (per mm), VA (>0.8, 0.5-0.8, 0.3-0.5, 0.05-0.3, and <0.05), and age (<45, 45-60, and ≥60 years) were categorized. he distribution of AL was assessed in the population-based versus case-control study samples. Pearson correlation coefficients (R^2)

were used to evaluate correlations between AL in right and left eyes, between AL and SE and between the AL/CC ratio and SE. The frequency of visual impairment was calculated per millimeter category of AL, stratified by age. We considered linear age trends on visual impairment using logistic regression, adjusting for gender. Cumulative risk of visual impairment (i.e., VA <0.3) was estimated per AL category using Kaplan-Meier product limit analysis. We assigned the age at diagnosis of blindness or impaired vision as the mean age between the examination in which this endpoint was observed and the previous examination. For participants who did not reach this endpoint, we used their age at the last examination for censoring. Participants who died or were otherwise lost to follow-up were analyzed using the last examination. All participants ≥85 years of age were censored at 80 years of age in order to ensure unbiased estimates. Statistical analyses were performed using the SPSS software package version 20.0 (IBM, Armonk, NY).

RESULTS

The general characteristics of our study populations are summarized in Table 1. We observed an increasing prevalence of myopia in our population-based Rotterdam studies due to a cohort effect described in our previous work[14,22]. The distributions of AL in the population-based studies and the MYST study are shown in Figure 1. The population-based studies (RS-I, RS-II, RS-III, and ERF) and the case-control study MYST included 131 and 684 right eyes with an AL ≥26 mm, respectively. The mean AL of all right eyes in the population-based studies was 23.38 mm (SD: 1.15); in the MYST study, the mean AL of the cases and controls was 27.36 mm (SD: 1.62) and 23.49 mm (SD: 0.76), respectively. High correlation was found in AL between right and left eyes (population-based R^2 = 0.906; case control R^2 = 0.932).

Figure 2 shows the correlation between AL and SE, and between the AL/CC ratio and SE. We found good correlation between AL and SE (population-based R^2 = 0.695; case-control R^2 = 0.906); and high correlation between SE and the AL/CC ratio (population-based R^2 = 0.798; case-control R^2 = 0.943), with the highest correlation at the more extreme ends of SE.

The relationship between AL and visual acuity in the three different age categories in the combined dataset is shown in Figure 3. Individual study data are presented in the Supplementary Information. We did not observe large differences in frequencies of visual impairment between the population-based studies and the case control study. The overall frequency of VI (i.e., VA <0.3) in our total study population was 1.5%. In subjects with high myopia the frequency of VI was 5.1%, which increased with age (*P* trend <0.0001). Among eyes from participants <45 years, 45-60 years, and ≥60 years of age, the frequency of VI was 2.6%, 5.3%, and 15% at AL ≥26 mm compared to 0%, 0.8%, and 0.8% at mean AL 23-24 mm, respectively.

Next, we examined the cumulative risk of VI in relation to AL; these data are shown in Figure 4. Our analysis revealed that the risk of VI was low (1.8%) in all AL categories at age 40; by age 80, the cumulative risk of VI was 7% (standard error of the mean (SEM): 0.029) for AL 23-24 mm, 30.5% (SEM 0.125) for AL 26-28, 44.7% (SEM: 0.171) for AL 28-30 mm, and 90.5% (SEM: 0.082) for AL ≥30 mm. The risk of VI for eyes with AL 26-28 mm increased gradually from 60-70 years, whereas eyes with AL >28 mm were visually impaired beginning at approximately 45 year of age.

Table 1. General characteristics of the study participants

	RS-I	RS-II	RS-III	ERF	MYST	
					cases	*controls*
N	662	1074	1148	2353	671	363
Gender (%) men	48.9	47.3	44.2	44.9	35.2	46.7
Age, years ± SD	60.5 ± 4.3	61.3 ± 4.6	59.6 ± 7.8	50.2 ± 12.7	46.0 ± 12.5	48.9 ± 12.5
Age, range	55.0 - 76.3	49.1 - 87.0	47.4 - 89.3	25.1 - 86.5	25.0 - 79.6	25.0 - 78.6
Age (years)						
< 45	0	0	0	872	278	116
45-60	342	488	647	913	275	169
≥60	320	586	501	568	118	78
Mean spherical equivalent, D (± SD)	0.50 ± 2.12	0.47 ± 2.49	-0.13 ± 2.45	0.12 ± 2.05	-10.4 ± 3.4	0.02 ± 0.28
Mean corneal curvature, radius (± SD)	7.73 ± 0.26	7.70 ± 0.24	7.75 ± 0.26	7.73 ± 0.27	7.74 ± 0.36	7.80 ± 0.25
Axial length (mm)						
Mean (± SD)	23.54 ± 1.07	23.50 ± 1.14	23.48 ± 1.32	23.25 ± 1.07	27.36 ± 1.62	23.50 ± 0.76
Range	20.8 - 27.65	19.79 - 29.17	16.33 - 30.12	19.66 - 29.70	23.41 - 33.46	21.17 - 25.82
< 22 mm (%)	5.9	8.1	9.6	10.3	0.1	2.2
22 - 23 mm (%)	24.5	23.8	26.8	31.2	0.0	24.8
23 - 24 mm (%)	38.5	39.2	33.7	37.7	0.4	43.3
24 - 25 mm (%)	22.1	20.2	18.2	15.5	3.7	26.4
25 - 26 mm (%)	6.9	5.7	7.9	3.6	14.9	2.8
26 - 27 mm (%)	1.8	2.4	3.0	1.4	28.3	0.3
27 - 28 mm (%)	0.3	0.5	0.3	0.3	22.4	0.3
28 - 29 mm (%)	0.0	0.0	0.1	0.0	13.7	0.0
29 - 30 mm (%)	0.0	0.1	0.3	0.0	5.8	0.0
30 - 31 mm (%)	0.0	0.0	0.1	0.0	4.9	0.0
31 - 32 mm (%)	0.0	0.0	0.0	0.0	3.6	0.0
32 - 33 mm (%)	0.0	0.0	0.0	0.0	1.2	0.0
≥ 33 mm (%)	0.0	0.0	0.0	0.0	0.9	0.0
Visual acuity						
Median visual acuity	1.00	1.00	0.64	1.00	1.00	1.25
> 0.8 (%)	96.5	93.9	NA	93.7	67.1	93.7
0.5 - 0.8 (%)	2.1	3.7	97.6	3.7	19.8	5.5
0.3 - 0.5 (%)	0.8	0.7	1.4	1.2	7.2	0.6
0.05 - 0.3 (%)	0.6	0.8	0.8	1.0	3.3	0.0
< 0.05 (%)	0.0	0.8	0.2	0.3	2.7	0.3

NA = not applicable

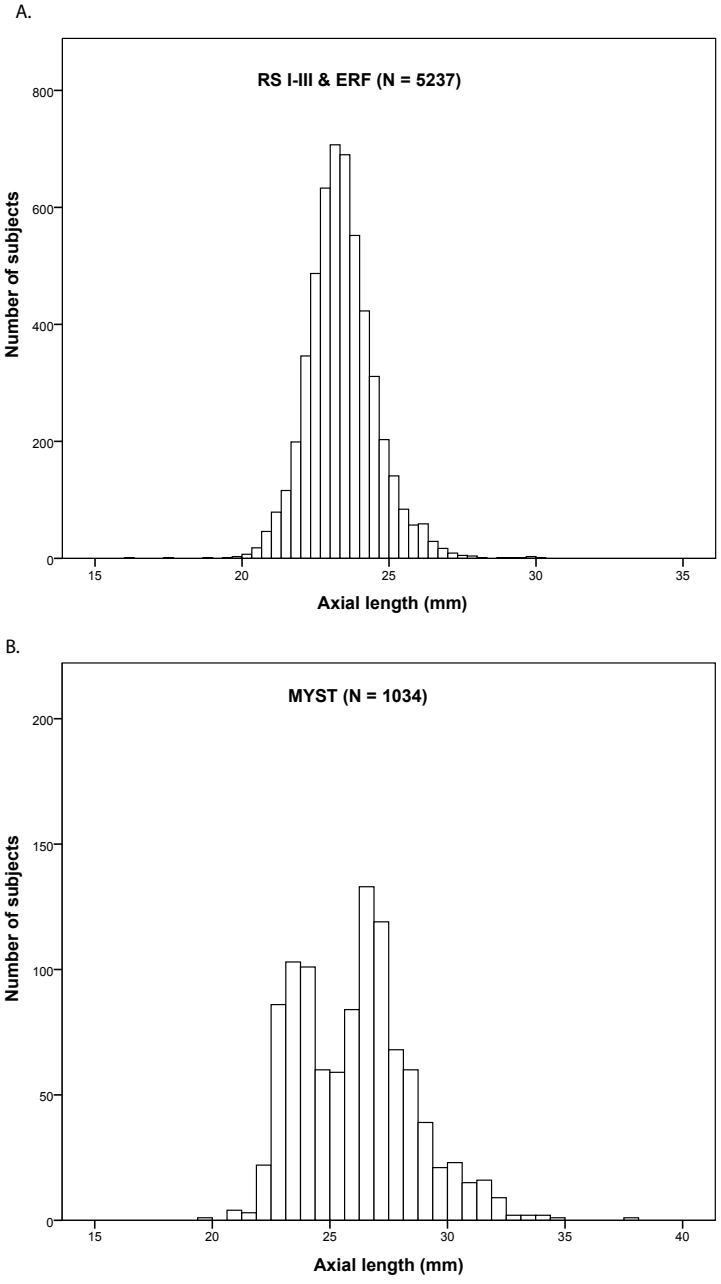


Figure 1. Distribution of axial length in the population-based studies A. and case-control study B.

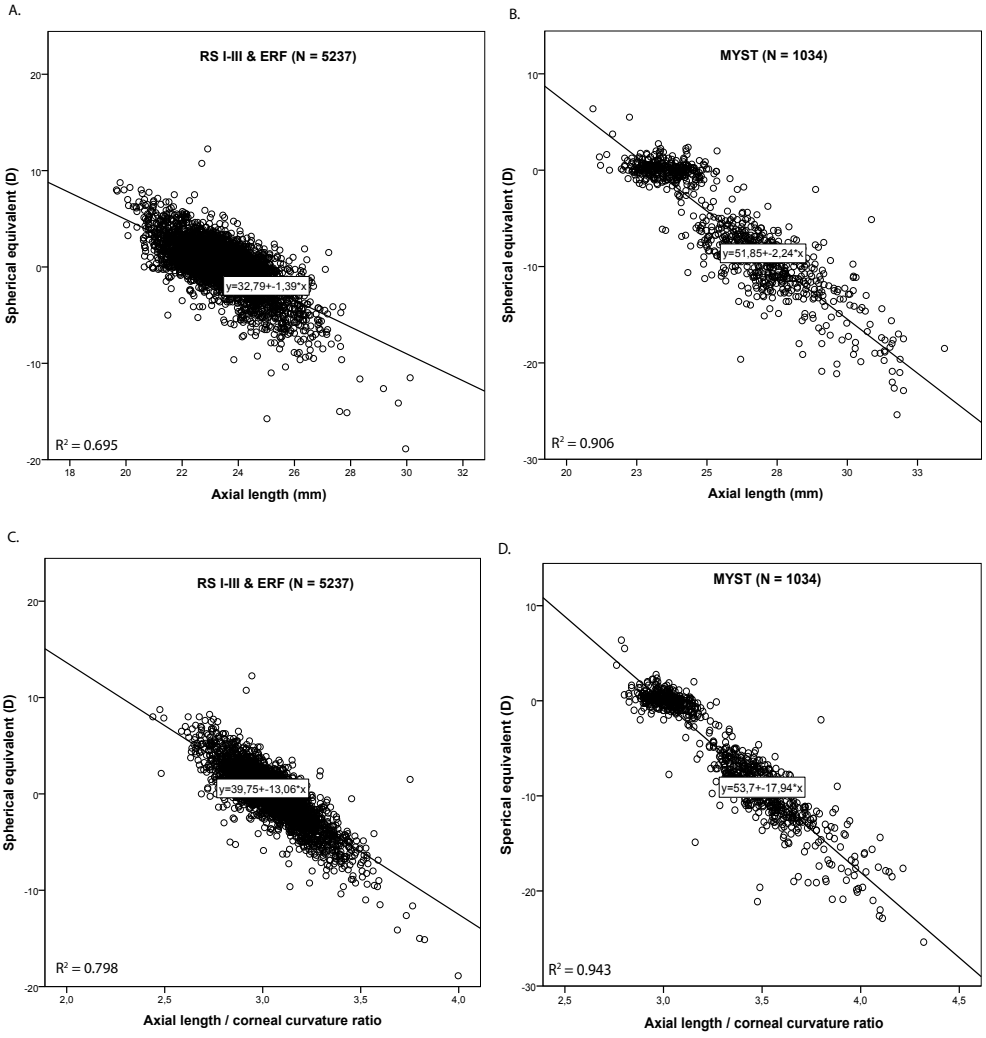


Figure 2. The correlations between spherical equivalent and axial length, and between spherical equivalent and the axial length/corneal curvature ratio

A. SE vs. AL in the population-based studies (RS I-III and ERF); B. SE vs. AL in the case-control study (MYST); C. SE vs. AL/CC ratio in the population-based studies (RS I-III and ERF); D. SE vs. AL/CC ratio in the case-control study (MYST)

DISCUSSION

In this study, we found a strong relationship between axial length and visual impairment in a large dataset which included a high number of high myopes. Strikingly, the cumulative risk of VI was 30% in eyes with AL 26-28 mm and 45% in eyes with AL 28-30 mm; in eyes with an AL>30 mm, this frequency increased to >90%. Eyes with AL 26-28 mm gradually developed VI from 60-70 years, whereas eyes with >28 mm began to develop VI as early as 45 years of age.

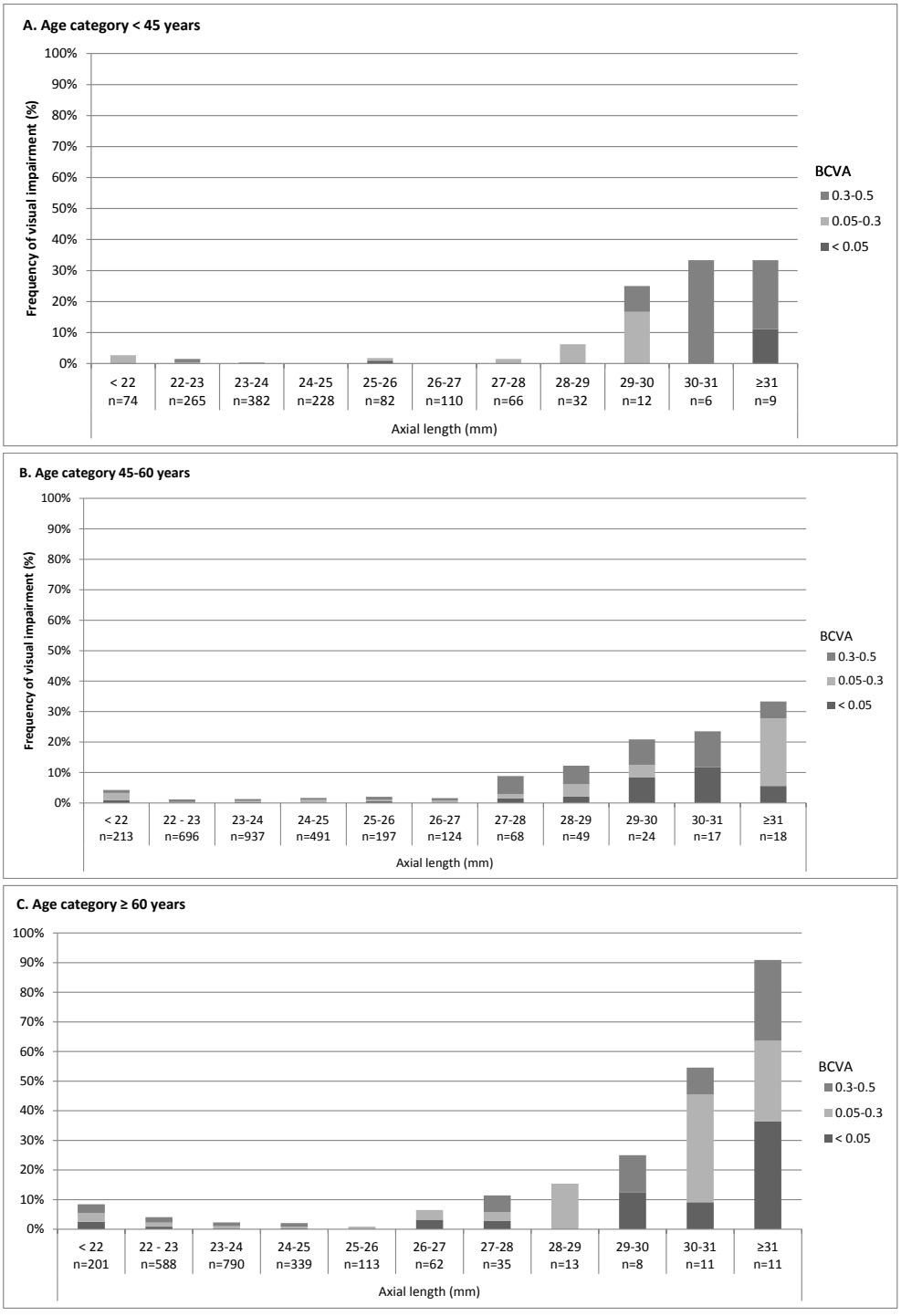
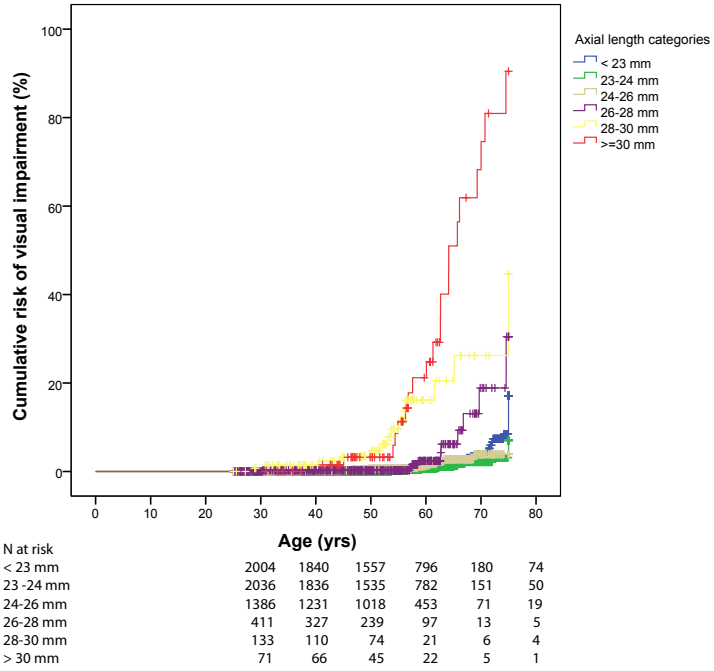


Figure 3. Frequency distribution of best-corrected visual acuity (BCVA), stratified by age and AL. A. < 45 years; B. 45-60 years; C. ≥60 years



N at risk						
< 23 mm	2004	1840	1557	796	180	74
23 -24 mm	2036	1836	1535	782	151	50
24-26 mm	1386	1231	1018	453	71	19
26-28 mm	411	327	239	97	13	5
28-30 mm	133	110	74	21	6	4
> 30 mm	71	66	45	22	5	1

Figure 4. Cumulative risk of visual impairment (WHO criteria) as a function of axial length

A strength of this study is that we combined our Rotterdam cohorts with a high myopia case-control study, to warrant a sufficiently high proportion of subjects at the extreme ends of AL. On the other hand, this combination was also a potential source of limitations. For example, selective non-participation of disabled persons in population-based studies may lead to underestimation of risks, while selective participation of functionally disabled in case control studies may overestimate this risk. The effect of these biases appeared to be small in our study, as risk estimates did not show statistical differences between the studies of different design.

Our findings are in line with previous studies which showed a higher prevalence of pathologic signs at greater axial lengths[11,23,24].Neither of these studies directly assessed the relationship between axial length and VA. We recently reported that one third of all high myopes eventually becomes bilaterally blind and/or visually impaired[14]. Although the number of high myopes in our previous study was relatively small, we already calculated a much higher risk of VI among individuals with a refractive error of -10 D compared to individuals with a refractive error between -6 and -10 D. Our current study describes in an even more detailed manner the exponential rise of risk of VI at extreme eye lengths.

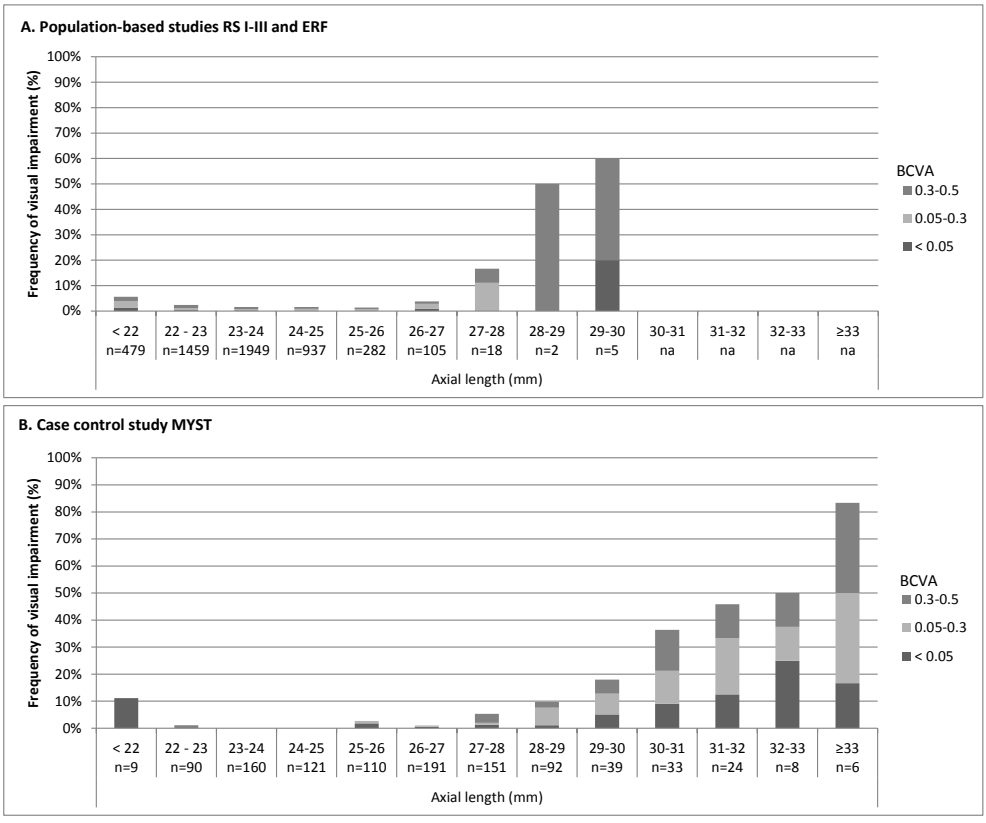
Our study suggests that age modifies the effect of AL on visual impairment. There appeared to be a lag between the onset of myopia, usually during childhood and adolescence, and the manifestation of visual impairment, which occurred predominantly in middle-aged and elderly adults. Other studies have also reported an age effect in relation to the occurrence of retinal

lesions and myopic retinopathy[25-28]. Experimental animal studies on the long term effects of myopia suggest that although the sclera thins rapidly during earlydevelopment, shifts in the collagen fibril diameter are not apparent until later in myopia development[29]. In experimental myopic chick eyes, neither staphyloma formation nor development of chorioretinal atrophy occurred soon after visual deprivation[30]. This suggests that, in mechanical stretching, age-related changes induce the development of myopic fundus changes. High myopic eyes with their thinned ocular tissue at the posterior pole may be particularly vulnerable for anatomical alterations that occur with aging, such as thickening of Bruch's membrane, vascular changes of the choroid, and scleral calcification[31].

Currently, few strategies are available for treating myopia. Atropine, a muscarinic receptor antagonist that can be applied topically to the eye, and orthokeratology lenses can be effective in inhibition of eye growth[32]. Our data underscore the value of these therapies even in high myopes, as achieving a lower final AL would significantly decrease the patient's risk of developing VI later in life. Likewise, preventative measures such as increasing outdoor exposure in progressive high myopes is warranted. Indeed, each incremental decrease in final AL increases the chance of lifelong preservation of visual acuity.

In summary, we examined the risk of VI in several categories of AL using data combined from population-based and case-control studies. The risk of VI in high myopes was highly correlated with AL and reached extreme values in AL ≥30 mm. Our data emphasize the morbidity of high myopia, and strengthen current strategies to develop treatment options.

SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Axial length in relation to best-corrected visual acuity (BCVA) in all age categories combined. A. Population-based studies RS I-III and ERF; B. Case control study MYST

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3.1

A genome-wide association study identifies a susceptibility locus for refractive errors and myopia at 15q14

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ABSTRACT

Refractive errors are the most common ocular disorders worldwide, and may lead to blindness. Although this trait is highly heritable, identification of susceptibility genes has been challenging. We conducted a genome-wide association study testing single nucleotide polymorphisms for association with refractive error in 5,328 unrelated individuals of a Dutch population-based study, and replicated findings in four independent cohorts (10,280 persons). We identified a significant association at chromosome 15q14 with $P=2.21 \times 10^{-14}$ for rs634990. The odds ratio of myopia versus hyperopia for the minor allele (MAF 0.47) was 1.41 (95% CI 1.16-1.70) for heterozygous, and 1.83 (95% CI 1.42-2.36) for homozygous subjects. The associated region lies in the vicinity of genes which are expressed in the retina, *GJD2* and *ACTC1*, and appears to harbor regulatory elements which may influence transcription of these genes. Our data suggest that common variants at 15q14 influence susceptibility for refractive errors in the general population.

INTRODUCTION & RESULTS

Refractive errors are by far the most common cause of visual impairment in humans[1-5]. They result from aberrant coordinated effects of the ocular biometric components, most notably of axial length. Elongation of the eye axis leads to myopia (nearsightedness), while a shortened axis causes hyperopia (farsightedness). Refractive errors often cause alterations in the anatomical structure of the eye, increasing the risk of complications[6]. Myopia may lead to ocular morbidity such as glaucoma and retinal detachment, and high myopia in particular can cause posterior staphyloma and macular degeneration[7-11]. Treatment options for myopia are limited; it is the fifth most common cause of impaired vision, and the seventh most common cause of legal blindness worldwide[10,11].

The etiology of refractive errors and myopia is complex and largely unknown. The current notion is that eye growth is triggered by a visually evoked signaling cascade, which begins in the retina, traverses the choroid, and subsequently mediates scleral remodeling. Established risk factors are education, reading, outdoor exposure, and familial predisposition[11-14]. Familial aggregation studies quantified a strong genetic basis; the estimated sibling recurrence risk (λ_s) varied between 1.5-3.0 for low myopia- and between 4.9-19.8 for high myopia, and heritability estimates (h^2) ranged from 0.60-0.90[15]. Segregation analyses suggested the involvement of multiple genes rather than a single major gene effect[11,13,15]. In an attempt to identify causal genes, previous mapping studies mainly focussed on highly myopic probands with multiple affected relatives, and thereby identified at least 20 putative genetic loci[11]. Replication of these results has been limited, and proposed loci were shown to have little to no effect in unselected populations. Genome-wide mapping has not been conducted in refractive error studies of the general population. Hence, the genetic basis of common refractive errors and myopia remains to be elucidated.

We performed a genome-wide association study (GWAS) in the population-based Rotterdam Study (RS-I, n=5328), and investigated refractive error as a quantitative trait. Study design and baseline characteristics are provided in the Methods and Supplementary Table 1. The mean spherical equivalent in this older population of European descent was +0.86 (standard deviation (SD) 2.45) dioptres. Refractive errors occurred in 52% (n=2790) of the participants, ranging from -19 to +10 diopters (D).

We genotyped the entire sample using the Illumina HumanHap 550k and 610Q arrays (Methods). Genotypes for more than 2.5 million autosomal single nucleotide polymorphisms (SNPs) were imputed with reference to the HapMap Phase II CEU build 36. Comparison of the observed and expected distributions (QQ plot, Supplementary Figure 1) showed modest inflation of the test statistics (λ_{GC}=1.054 for RS-I). Using an additive model, we identified a novel genome-wide significant (P=1.76x10^{-8}) locus on chromosome 15q14 (Table 1, Figure 1). Subsequently, we investigated 31 SNPs spread across four loci on chromosome 15q14, 14q24, 1q41, and 10p12.3 reaching P<10^{-6} (Supplementary Table 2) for further investigation in four independent replication cohorts, i.e., RS-II (n=2008; λ_{GC}=1.012), RS-III (n=1970; λ_{GC}=1.012), Erasmus Rucphen Family Study (ERF, n=2032; λ_{GC}=1.037) from the Netherlands; and a twin study from the United Kingdom (TwinsUK; n=4270; λ_{GC}=1.04. The designs of RS-II and RS-III were population-based; those of ERF and TwinsUK family-based. Cohorts were not selected on a disease phenotype. All studies

Table 1. Genome-wide association and replication for refractive error at locus 15q14

		Discovery cohort: RS-I (n = 5328)				Replication RS-II (n = 2008)		RS-III (n = 1970)		ERF (n = 2032)		TwinsUK (n = 4270)		Meta-analysis (n = 15608)	
SNP	Position	MA	MAF	Beta (sem)	P	Beta (sem)	P	Beta (sem)	P	Beta (sem)	P	Beta (sem)	P	Beta (sem)	P
rs688220	32786167	A	0.45	-0.27 (0.05)	$1.76x10^{-8}$	-0.28 (0.08)	$3.80x10^{-4}$	-0.22 (0.08)	$9.27x10^{-3}$	-0.03 (0.07)	$6.24x10^{-1}$	-0.15 (0.07)	$2.60x10^{-2}$	-0.20 (0.0009)	$2.79x10^{-11}$
rs580839	32786121	A	0.44	-0.27 (0.05)	$1.89x10^{-8}$	-0.27 (0.08)	$4.96x10^{-4}$	-0.22 (0.08)	$7.95x10^{-3}$	-0.03 (0.07)	$6.34x10^{-1}$	-0.16 (0.07)	$1.92x10^{-2}$	-0.20 (0.0009)	$2.53x10^{-11}$
rs619788	32782398	A	0.44	-0.27 (0.05)	$1.92x10^{-8}$	-0.27 (0.08)	$4.94x10^{-4}$	-0.22 (0.08)	$7.72x10^{-3}$	-0.03 (0.07)	$6.27x10^{-1}$	-0.16 (0.07)	$1.85x10^{-2}$	-0.20 (0.0009)	$2.53x10^{-11}$
rs4924134	32781857	G	0.44	-0.27 (0.05)	$2.04x10^{-8}$	-0.27 (0.08)	$4.76x10^{-4}$	-0.27 (0.08)	$6.58x10^{-3}$	-0.06 (0.07)	$4.10x10^{-1}$	-0.16 (0.07)	$1.85x10^{-2}$	-0.21 (0.0009)	$1.36x10^{-12}$
rs560766	32788234	A	0.44	-0.26 (0.05)	$4.27x10^{-8}$	-0.28 (0.08)	$4.54x10^{-4}$	-0.21 (0.08)	$1.29x10^{-2}$	-0.03 (0.07)	$6.65x10^{-1}$	-0.18 (0.07)	$7.68x10^{-3}$	-0.20 (0.0009)	$2.49x10^{-11}$
rs7176510	32786771	T	0.45	-0.26 (0.05)	$5.16x10^{-8}$	-0.28 (0.08)	$5.10x10^{-4}$	-0.22 (0.08)	$9.62x10^{-3}$	-0.02 (0.07)	$7.51x10^{-1}$	-0.16 (0.07)	$1.76x10^{-2}$	-0.20 (0.0009)	$6.25x10^{-11}$
rs7163001	32777866	A	0.44	-0.26 (0.05)	$5.23x10^{-8}$	-0.28 (0.08)	$4.08x10^{-4}$	-0.23 (0.08)	$5.89x10^{-3}$	-0.07 (0.07)	$3.01x10^{-1}$	-0.16 (0.07)	$1.87x10^{-2}$	-0.21 (0.0009)	$5.61x10^{-12}$
rs11073060	32777143	A	0.44	-0.26 (0.05)	$5.76x10^{-8}$	-0.28 (0.08)	$4.05x10^{-4}$	-0.23 (0.08)	$5.82x10^{-3}$	-0.08 (0.07)	$2.72x10^{-1}$	-0.16 (0.07)	$1.91x10^{-2}$	-0.21 (0.0009)	$3.65x10^{-12}$
rs8032019	32778782	G	0.40	-0.26 (0.05)	$6.09x10^{-8}$	-0.28 (0.08)	$5.57x10^{-4}$	-0.13 (0.09)	$1.30x10^{-1}$	-0.05 (0.07)	$5.12x10^{-1}$	-0.16 (0.07)	$1.96x10^{-2}$	-0.19 (0.0009)	$3.71x10^{-10}$
rs685352	32795627	G	0.44	-0.25 (0.05)	$8.80x10^{-8}$	-0.25 (0.08)	$1.28x10^{-3}$	-0.19 (0.08)	$1.98x10^{-2}$	-0.07 (0.07)	$3.06x10^{-1}$	-0.24 (0.07)	$4.43x10^{-4}$	-0.21 (0.0009)	$4.19x10^{-12}$
rs524952	32793178	A	0.47	-0.25 (0.05)	$1.03x10^{-7}$	-0.30 (0.08)	$2.09x10^{-4}$	-0.19 (0.08)	$2.56x10^{-2}$	-0.06 (0.07)	$4.13x10^{-1}$	-0.32 (0.07)	$4.15x10^{-6}$	-0.23 (0.0009)	$3.18x10^{-14}$
rs634990	32793365	C	0.47	-0.25 (0.05)	$1.03x10^{-7}$	-0.30 (0.08)	$2.15x10^{-4}$	-0.20 (0.08)	$2.03x10^{-2}$	-0.05 (0.07)	$5.11x10^{-1}$	-0.33 (0.07)	$2.93x10^{-6}$	-0.23 (0.0009)	$2.21x10^{-14}$
rs11073059	32776966	A	0.44	-0.25 (0.05)	$1.20x10^{-7}$	-0.28 (0.08)	$3.96x10^{-4}$	-0.23 (0.08)	$5.83x10^{-3}$	-0.08 (0.07)	$2.72x10^{-1}$	-0.16 (0.07)	$1.91x10^{-2}$	-0.20 (0.0009)	$8.45x10^{-12}$
rs11073058	32776918	T	0.44	-0.25 (0.05)	$1.30x10^{-7}$	-0.28 (0.08)	$3.93x10^{-4}$	-0.23 (0.08)	$5.84x10^{-3}$	-0.08 (0.07)	$2.71x10^{-1}$	-0.16 (0.07)	$1.90x10^{-2}$	-0.20 (0.0009)	$8.45x10^{-12}$

RS-I, Rotterdam Study I; RS-II, Rotterdam Study II; RS-III, Rotterdam Study III; ERF, Erasmus Rucphen Family Study; TwinsUK, the Twin Cohort recruited in London; SNP, single nucleotide polymorphism; MA, Minor Allele; MAF, Minor Allele Frequency; Beta, effect size on spherical equivalent in diopters; sem, standard error of the mean.

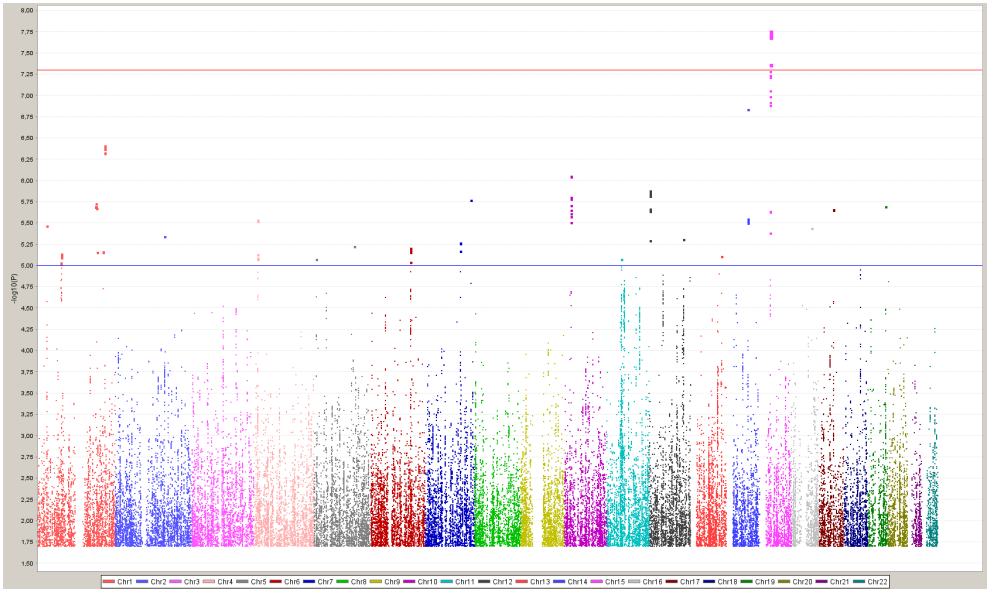

Figure 1. Genome-wide signal intensity (Manhattan) plot of discovery cohort Rotterdam Study I
The statistical significance values across the 22 autosomes of each SNP association with refractive error (measured as spherical equivalent) are plotted as $-\log_{10}$ *P*-values. SNPs with minor allele frequency >=0.01 were included. The blue horizontal line indicates *P*-value of 10^{-5}; the red line *P* value of $5x10^{-8}$.

consisted predominantly of individuals of European ancestry, and all used similar protocols to evaluate refractive error (Methods, Supplementary Table 2).

At validation, meta-analysis confirmed a significant association between refractive errors and locus 15q14 (Table 1). Frequencies of the risk alleles at this region were similar across the studies. The *P*-values were nominally significant for the 14 top SNPs in RS-II, RS-III, and TwinsUK, and the direction of the effect (regression coefficient beta) of the minor alleles was consistent. The strongest signal in the meta-analysis was observed for rs634990 ($P=2.21x10^{-14}$; Table 1), and this SNP accounted for 0.5% of the variance in spherical equivalent.

To determine the impact of this locus on the risk of clinically relevant outcomes, we compared subjects with myopia to those with hyperopia in a logistic regression analysis. We found strong evidence that the C allele of rs634990 carried a higher risk of myopia (Figure 2). The odds ratio (OR) of mild or severe myopia versus mild or severe hyperopia was 1.41 (95% Confidence Interval (CI) 1.16-1.70) for heterozygous individuals, and 1.83 (95% CI 1.42-2.36) for homozygous persons.

The 15q14 region of highly significant SNPs (Figure 3) lies in an intergenic region in the vicinity of the genes *GJD2* (39 kb from rs634990 at 3' end), *ACTC1* (74 kb at 3'end), and *GOLGA8B* (180 kb at 5'end). We investigated a potential function for these genes in eye growth development by examining gene expression levels in the retina of postmortem human eyes (Supplementary Table 3), and observed a moderate to high expression for *GJD2* and *ACTC1,* and a much lower expression for *GOLGA8B*. *GOLGA8B* (Golgi autoantigen golgin-67) encodes a 67 kDa protein,

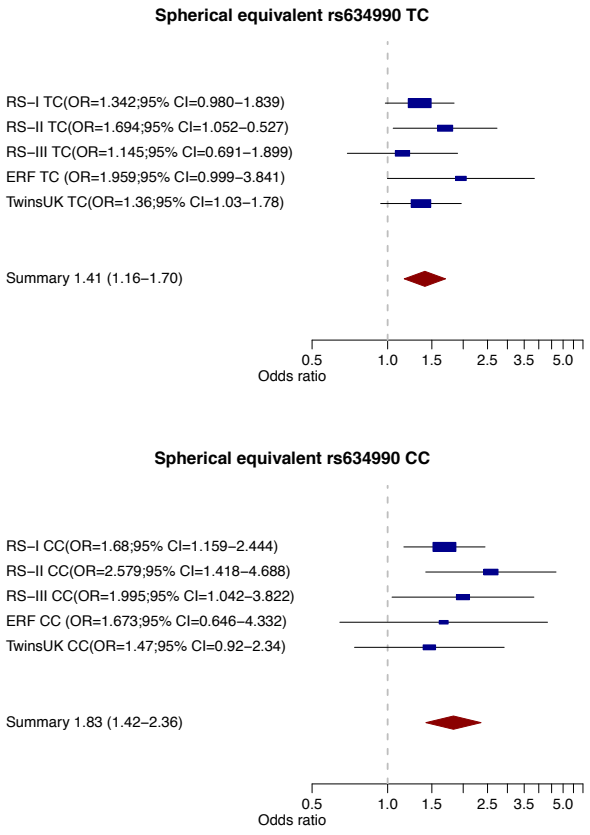

Figure 2. Forest plot of associations for myopia (SE<= -3D) versus hyperopia (SE >= +3D)
Forest plot of the estimated per-genotype odds ratio for topSNP rs634990 for the 5 studies separately, and for the meta-analysis of all studies. RS-I, Rotterdam Study I; RS-II, Rotterdam Study II; RS-III, Rotterdam Study III; ERF, Erasmus Rucphen Family Study; TwinsUK, the Twin Cohort recruited in the UK; OR, odds ratio; 95%CI, 95% Confidence Interval.

belongs to a family of Golgi auto-antigens, and is localized at the cytoplasmic surface of the Golgi complex[16]. A specific function of this gene in the retina has not been reported. *ACTC1* (cardiac muscle alpha actin 1) encodes a 42 kDa smooth muscle actin. The functional role of *ACTC1* in the eye is currently unclear, but actins which are similar, such as α-SMA, have been shown to be increased in developing myopic eyes[17]. α-SMA influences the number of contractile myofibroblasts in the sclera, and contributes to extracellular matrix remodeling. As these are key factors occurring in eye enlargement, it is intriguing to know whether *ACTC1* has these characteristics as well.

The functional properties of *GJD2* make this gene an interesting candidate to explain our findings. *GJD2* (gap junction protein delta 2) encodes the 36 kDa connexin36 (CX36), which is a neuron-specific protein belonging to a multi-gene family of integral membrane proteins[18]. CX36 forms gap junction channels between adjacent membranes of neuronal cells, is present in photoreceptors, amacrine, and bipolar cells, and plays a critical role in the transmission process of the retinal electric circuitry by enabling intercellular transport of small molecules and ions[18-21]. Further exploration of

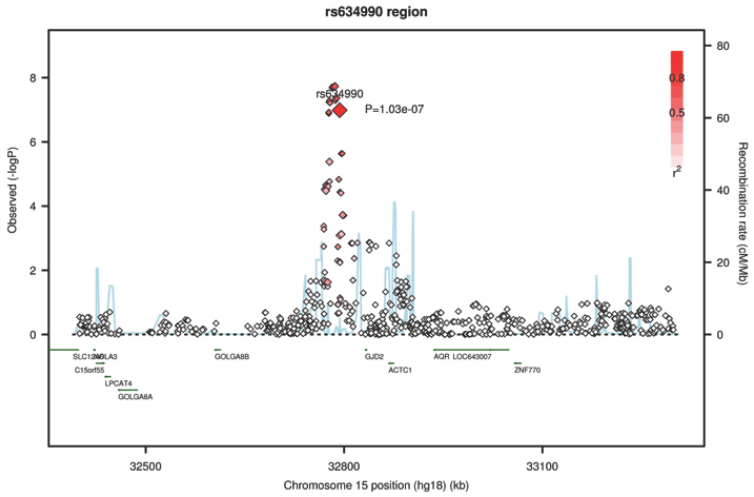

Figure 3. Regional plot at chromosome 15q14
Log_{10} *P*-values from the discovery cohort Rotterdam Study I as a function of genomic position (HapMap release 22 build 36). The *P*-value for the top SNP is denoted by the large diamond; *P*-values for other genotyped and imputed SNPs are shown as smaller diamonds. *P*-values for SNPs of unknown type are presented as squares. Superimposed on the plot are gene locations (green) and recombination rates (blue).

GJD2 using Ingenuity analysis (Methods, Supplementary Figure 2) alluded to a role in eye growth regulation as well as lens fiber maturation in knock-out animals[22,23]. To identify possible causal variants in this gene, we performed direct sequencing of all exons and intron-exon boundaries of *GJD2* in 47 subjects with either high myopia, high hypermetropia, or emmetropia. We found neither new mutations nor frequency differences of variants between groups (Supplementary Table 4), and conclude that linkage disequilibrium with common functional variants in *GJD2* is unlikely to explain the observed association.

The next step was to assess whether the intergenic region itself can have functional consequences. We evaluated the expression of SNPs of our associated region in lymphoblastoid cell lines. At least two of our most associated SNPs significantly altered expression, providing evidence that elements of our locus are transcribed and may alter cell function (Supplementary Table 5). Subsequently, we searched for regulatory elements[24,25] in the entire 53 kb locus of highly significantly correlated SNPs using UCSC Genome Browser, and found the predicted presence of seven DNase I hypersensitive sites, six enhancers based on experimentally validated H3 chromatin signatures in Hela and K562 cells[24,25], 20 peaks of sequence conservation in alignments of multiple species of placental mammals, and one insulator site (Supplementary Figure 3)[25]. Enhancers are known to facilitate transcription of distal genes, and its range of activity is confined by insulators[25]. Remarkably, the greatest peak of our association coincided with an insulator site. Precedents of genomic alterations of insulators causing hereditary disease have been reported[26,27]. We speculate that variants or mutations in regulatory elements at 15q14 may lead to illegitimate transcription of genes in the area, e.g., of *ACTC1* and *GJD2*.

In GWA studies, sources of heterogeneity may cause spurious findings. To address this issue and minimize potential biases, we applied genomic control to the cohort-level test statistics in the population cohorts, and correction using the identity by descent structure for the family-based cohorts. Three studies significantly replicated our initial findings. The fourth study, ERF, showed the same direction of association, albeit non-significant, and revealed similar risks of myopia for carriers of the risk allele (Figure 2). Thus, the observed effects of the genetic variants at 15q14 are relatively homogeneous among the 5 studies, enhancing credibility of the findings.

In the same issue of this journal, Hysi et al. report the results of a GWAS for refractive errors in the TwinsUK study[28]. The authors find genome-wide significance (best combined $P=1.85x10^{-9}$ for rs939658 and $P=2.07x10^{-9}$ for rs8027411) for a locus on chromosome 15q25, explaining 0.81% of the variance in spherical equivalent. The locus includes the promoter of the *RASGRF1* gene. This gene is known to be functionally involved in eye development[29], and, similar to *GJD2*, is involved in synaptic transmission of photoreceptor responses[30]. TwinsUK and RS-I are two of the largest existing refractive error cohorts with GWAS data. Our studies identified different genome-wide significant tophits in terms of *P*-values, and we both estimated the variation in refractive error explained by these SNPs to be small. Therefore, it is likely that common variants with a substantial disease risk do not play a role in the pathogenesis of this trait. The findings of our studies suggest that the genetic variance of refractive error is mostly determined by multiple variants with a low to moderate penetrance, resembling traits such as height[31].

Nevertheless, the mutual validation of the direction and beta of the effect of variants at 15q14 and 15q25 suggests that alterations at these genomic loci lead to refractive error and myopia. To unravel the mechanism, next steps should include comprehensive resequencing of the entire associated regions and flanking genes, validation in cohorts of other ethnicities, functional assays, and study of risk modulation by environmental factors. This may help to launch new pathogenic pathways for refractive errors, and may eventually lead to novel strategies to reduce the sight-threatening consequences of myopia.

MATERIAL & METHODS

Discovery cohort

The Rotterdam Study (RS-I) is a prospective population-based cohort study of 7,983 residents aged 55 years and older living in Ommoord, a suburb of Rotterdam, the Netherlands[32]. The baseline examination for the ophthalmic part took place between 1991 and 1993, and included 6,775 persons. Subjects were excluded if they had undergone bilateral cataract surgery, laser refractive procedures, or other intra-ocular procedures which might alter refraction. Complete data on refractive error and genome-wide SNPs were available on 5,328 persons, of whom 99% were of European ancestry.

Replication cohorts

The first three replication studies originated from the Netherlands. The first cohort was RS-II, an independent cohort which included 2,157 new participants aged 55+ years living in Ommoord

since 2000[32], who had good quality genotyping data. Baseline examinations took place between 2000 and 2002; follow-up examination from 2004 to 2005. The second replication cohort was RS-III, a study which included 2,082 new participants aged 45 and older living in Ommoord since 2006, who had good quality genotyping data. Baseline examination took place between 2006 and 2009. The third replication study was the Erasmus Rucphen Family (ERF) Study, a family-based study in a genetically isolated population in the southwest of the Netherlands. This study included 2,032 living descendants aged 18 years and older originating from 22 families who had at least six children baptized in the community church between 1880 and 1900, and who had good quality genotyping data. The fourth replication cohort was derived from the United Kingdom (TwinsUK). This study is an adult twin registry of over 10,000 healthy volunteer twins based at St Thomas' Hospital in London. Participants were recruited and phenotyped between 1998 and 2008. A total of 4,270 Caucasian participants had complete data on ocular phenotype and genotype[33].

As in the discovery cohort, participants in the four replication cohorts had been excluded if they had undergone bilateral surgery which inhibited evaluation of the original refractive error.

Measurements of refractive error

All studies used a similar protocol for phenotyping. Participants underwent an ophthalmologic examination which included non-dilated automated measurement of refractive error (RS I –III, ERF: Topcon RM-A2000 autorefractor; TwinsUK cohort: Humphrey-670 (Humphrey Instruments, San Leandro, CA) from 1998 to 2002; and then ARM-10 (Takagi Seiko, Japan), best-corrected visual acuity, and keratometry. Spherical equivalent (SE) was calculated from the standard formula: spherical equivalent = sphere + (cylinder/2). In addition to investigating SE as a quantitative trait, we stratified SE into categories of refractive error to evaluate findings from a clinical viewpoint. Myopia was categorized into low (SE –1.5 to –3 diopters (D)), moderate (SE –3 to –6D), and high (SE –6 D or lower). For hyperopia, these categories were mild (SE +1.5 to +3D), moderate (SE +3 to +6D), and high (SE +6D or higher), respectively. We considered SE -1.5 to +1.5D as emmetropia.

Ethics

All measurements in RS-I-III and ERF were conducted after the Medical Ethics Committee of the Erasmus University had approved the study protocols, and all participants had given a written informed consent in accordance with the Declaration of Helsinki. In the TwinsUK study, all twins gave fully informed consent under a protocol reviewed by the St Thomas' Hospital Local Research Ethics Committee.

Genotyping

Discovery cohort

All persons attending the baseline examination in 1990-1993 consented to genotyping, and had DNA extracted from blood leucocytes. Genotyping of autosomal SNPs was performed in persons with high-quality extracted DNA (n=6,449) using the Illumina Infinium II HumanHap550chip v3.0®

array according to the manufacturer's protocols. Samples with low call rate (<97.5%, n=209), with excess autosomal heterozygosity (>0.336, n=21), and with sex-mismatch (n=36) were excluded, as were outliers identified by the identity-by-state (IBS) clustering analysis (>3 standard deviations from population mean, n=102 or IBS probabilities >97%, n=129). The total sample of individuals with good quality genotyping data was 5,974.

Replication cohorts

In RS-II, the majority of the 2,516 DNA samples were genotyped using the HumanHap 550 Duo Arrays; 133 (5%) were genotyped using the Human 610 Quad Arrays (Illumina). In the RS-III cohort, all DNA samples were genotyped using the Illumina Infinium II HumanHap550chip v3.0® array. In ERF, DNA was genotyped on four different platforms (Illumina 6k, Illumina 318K, Illumina 370K and Affymetrix 250K). Genotyping for the TwinsUK cohort took place in stages; in the first stage 1,810 individuals were genotyped using Illumina's HumanHap 300k duo chip, at a later stage 2,578 persons were genotyped using Illumina's HumanHap610 Quad.

Imputation

The set of genotyped input SNPs used for imputation in each study was selected based on highest quality GWA data. The callrate was set at >98% in Rotterdam Study I-III; the minor allele frequency at >0.01; and the Hardy-Weinberg $P > 10^{-6}$. We used the Markov Chain Haplotyping (MaCH) package version 1.0.15 software (Rotterdam; imputed to plus strand of NCBI build 36, HapMap release #22) for the analyses. For each imputed SNP, a reliability of imputation was estimated (as the ratio of the empirically observed dosage variance to the expected binomial dosage variance: O/E ratio).

Statistical analysis

Discovery cohort

Refractive error measured at baseline as a continuous variable was used as outcome in the analysis. We calculated the mean SE for those with measurements on both eyes, and included the SE of only one eye if data from the other eye were missing. Linear regression models with 1-degree of freedom trend test were used to examine the associations between SNPs and SE, adjusted for age and gender. Using these linear regression models, we calculated regression coefficients with corresponding 95% confidence intervals (CIs). Odds ratios (ORs) of myopia and hyperopia were calculated with logistic regression analysis, adjusting for age and gender. GWAS analyses were performed using GRIMP[34].

We used genomic control to obtain optimal and unbiased results, and applied the inverse variance method of each effect size estimated for both autosomal SNPs that were genotyped and imputed in both cohorts. A *P*-value $<5 \times 10^{-8}$ was considered genome-wide significant.

Replication analyses

The topSNPs with *P*-value $<1 \times 10^{-6}$ from the discovery analysis were examined in the replication cohorts RS-II, RS-III, ERF and TwinsUK cohorts using SPSS version 15.0.0 for Windows (SPSS inc., Chicago, IL, USA; 2006), and R statistical package version 2.8.1 for Linux. A meta-analysis was

performed on all 5 studies using Metal for Linux.

GRIMP[34] was used for the analysis of the population-based replication cohorts. To adjust for family relationships, the GenABEL package[35] was used in the ERF study, and Merlin in the TwinsUK Study[36]. SNPs which deviated significantly from Hardy-Weinberg equilibrium ($P<10^{-6}$), or which had minor allele frequency <0.01 were excluded.

Gene expression data in human eye tissue

Human gene expression data were obtained essentially as described[37]. In short, postmortem eye bulbs (RPE: 6 donor eyes, choroid: 3 donor eyes, photoreceptors: 3 donor eyes), provided by the Corneabank Amsterdam, were rapidly frozen using liquid N2. Donors were between 63 and 78 years old and had no known history of eye pathology.

Cryosections were cut from the macula, and histology confirmed a normal histological appearance. RPE, photoreceptor and choroidal cells were isolated from macular sections using a Laser Microdissection System (PALM, Bernried, Germany). Total RNA was isolated and the mRNA component was amplified, labelled, and hybridized to a 44k microarray (Agilent Technologies, Amstelveen, The Netherlands)[38]. At least 3-6 microarrays were performed per tissue. Sample isolation, procedures, and expression microarray analysis were carried out according to obligatory MIAMI guidelines and the relevant expression data are deposited in the GEO database (2010) with accession number GSE20191. As a measure of the level of expression we sorted all the genes represented on the 44k microarray by increasing expression and calculated the corresponding percentiles (Supplementary Table 3).

Ingenuity database search

We explored the Ingenuity knowledge database using the keyword 'eye development' for all genes involved in 'function or diseases'. This search provided approximately 100 genes, which formed a new network for eye development. We subsequently added the *GJD2* gene to the network, and used the Path Explorer tool to search for possible functional relationships between *GDJ2* and these eye development genes in human, mouse, rat, and in vitro models (Supplementary Figure 2A). We continued the search using the keyword 'eye growth' for all genes involved in 'function or diseases', and investigated functional links between molecules using the connect tool and upstream-downstream analysis (Supplementary Figure 2B).

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3.2

Large scale international replication and meta-analysis study confirms association of the 15q14 locus with myopia: the CREAM consortium

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ABSTRACT

Myopia is a complex genetic disorder and a common cause of visual impairment among working age adults. Genome-wide association studies have identified susceptibility loci on chromosomes 15q14 and 15q25 in Caucasian populations of European ancestry. Here, we present a confirmation and meta-analysis study in which we assessed whether these two loci are also associated with myopia in other populations. The study population comprised 31 cohorts from the Consortium of Refractive Error and Myopia (CREAM) representing 4 different continents with 55,177 individuals; 42,845 Caucasians and 12,332 Asians. We performed a meta-analysis of 14 single nucleotide polymorphisms (SNPs) on 15q14 and 5 SNPs on 15q25 using linear regression analysis with spherical equivalent as a quantitative outcome, adjusted for age and sex. We calculated the odds ratio (OR) of myopia versus hyperopia for carriers of the top-SNP alleles using a fixed effects meta-analysis. At locus 15q14, all SNPs were significantly replicated, with the lowest *P*-value 3.87 x 10^{-12} for SNP rs634990 in Caucasians, and 9.65 x 10^{-4} for rs8032019 in Asians. The overall meta-analysis provided P-value 9.20 x 10^{-23} for the top SNP rs634990. The risk of myopia versus hyperopia was OR 1.88 (95% CI 1.64, 2.16, P < 0.001) for homozygous carriers of the risk allele at the top SNP rs634990, and OR 1.33 (95% CI 1.19, 1.49, P < 0.001) for heterozygous carriers. SNPs at locus 15q25 did not replicate significantly (*P*-value 5.81 x 10^{-2} for top SNP rs939661). We conclude that common variants at chromosome 15q14 influence susceptibility for myopia in Caucasian and Asian populations world-wide.

INTRODUCTION

Refractive errors are common optical defects of the visual system. An important refractive error is myopia (nearsightedness), which occurs when the eye elongates beyond the focal plane. The prevalence of myopia is high, affecting about one third of the world's population, and reaching over 70% in certain Asian ethnic groups[1-5]. High degrees of myopia are associated with pathologic ocular changes, such as myopic macular degeneration, retinal detachment, and glaucoma[6-10]. Due to the limited treatment options, myopia is a common cause of visual impairment[10,11].

Refractive errors, and myopia in particular, are complex genetic traits with a largely unknown etiology. Established environmental factors are education, early reading, and reduced outdoor exposure[11-17]. Although heritability estimates are high (50-90%[18]), the search for myopia genes is still ongoing. Previous linkage and association studies have led to the identification of at least 18 myopia (MYP) loci, 10 additional chromosomal regions, and several candidate genes[11,19]. Replication of these associations has been inconsistent, and their application to the general population is limited[19].

Recent genome-wide association studies (GWAS) reported several susceptibility loci for refractive error and myopia[20-25]. Solouki et al.[25] and Hysi et al.[20] were the first to perform a GWAS in a general Caucasian population, and identified susceptibility loci on chromosomes 15q14 and 15q25, respectively. In both studies, carriers of single nucleotide polymorphism (SNP) rs634990 at 15q14 (OR 1.83, 95% CI 1.42-2.36) and of SNP rs8027411 at 15q25 (OR 1.16, 95% CI 1.02–1.28) had a higher risk of myopia. Confirmation of these findings was obtained in various replication studies[20,25,26]. However, these replication cohorts were relatively limited in size, increasing the chance of a type 1 error.

To address potential inaccuracies and to investigate generalizability, we investigated the associations between refractive error and the 15q14 and 15q25 susceptibility loci in a large international replication and meta-analysis study (Consortium of Refractive Error and Myopia, CREAM) including 31 cohorts with various ethnicities from 4 different continents.

RESULTS

Meta-analysis of allelic effects on spherical equivalent (SE)

Complete data on refractive error and genome-wide SNPs were available in all 29 population-based studies comprising 49,364 subjects: 42,224 Caucasians and 7,140 Asians (Table 1, Figure 1, Supplementary Table 1). This includes the previously reported discovery set consisting of 15,608[25] and 17,608 subjects[20], respectively.

Table 1. Descriptives of all study cohorts

Study	n	mean age (SD)	age range	% men	mean SE (SD)
1958 British Birth Cohort	1658	42 (0.0)	40-50	54.2%	-0.96 (2.00)
AGES Reykjavik	2986	76.3 (5.4)	60-80+	35.3%	1.22 (2.05)
ALSPAC	3804	15.4 (0.3)	14.25-17.08	47.2%	-0.38 (1.28)
AREDS 1	816	79.5 (5.1)	60-80+	43.5%	0.68 (1.94)
AREDS 2	1506	68.0 (4.7)	55-81	41.1%	0.54 (2.25)
Australian Twins	1819	22.2 (12.7)	5-90	44.0%	-0.22 (1.28)
BMES	1574	64 (7.9)	50-80+	43.4%	0.59 (1.96)
Croatia Split	366	49.8 (14.4)	18-85	46.0%	-1.83 (1.83)
Croatia Vis Island	544	55.8 (14.0)	18-83	40.0%	-0.16 (1.93)
Croatia Korcula Island	836	56.0 (13.8)	18-98	35.0%	-0.25 (1.92)
ERF	2032	48.5 (14.3)	18+	43.1%	0.07 (2.13)
EGCUT	338	34.8 (15.2)	18-85	36.9%	-2.60 (2.00)
Finnish Twin Study on Aging	127	68.2 (3.8)	63-76	0.0%	1.68 (1.54)
Framingham Eye Study	1500	55.5 (9.0)	20-80	42.5%	-0.17 (2.40)
Gutenberg Health Study I	2745	55.7 (11)	35-74	51.5%	-0.38 (2.44)
Gutenberg Health Study II	1142	55.0 (10.9)	35-74	49.8%	-0.41 (2.58)
KORA	1867	55.6 (11.7)	35-84	49.6%	-0.29 (2.27)
MESA	1462	62 (9.4)	46-86	49.5%	-0.28 (2.62)
ORCADES	505	54.8 (13.7)	22-88.5	43.0%	0.01 (2.14)
Rotterdam Study I	5328	68.5 (8.6)	55+	41.3%	0.86 (2.45)
Rotterdam Study II	2009	64.2 (7.4)	55+	45.9%	0.48 (2.51)
Rotterdam Study III	1970	56.0 (5.5)	45+	43.9%	-0.35 (2.62)
OGP Talana	623	44.5 (21.1)	5-89	51.8%	-0.15 (1.78)
SCORM	929	10.8 (0.8)	10-15	48.0%	-2.02 (2.26)
SiMES	2226	57.7 (10.8)	40-80	49.3%	-0.08 (1.98)
SINDI	2055	55.7 (8.7)	40-80+	51.2%	0.01 (2.13)
SP2	1930	47.5 (10.9)	20-80	45.4%	-1.67 (2.89)
TwinsUK	4270	55.0 (12.0)	20-82	7.4%	-0.39 (2.73)
Young Finns	397	37.6 (5.2)	25-50	45.0%	-1.20 (2.29)
Kyoto Study	5192	NA	NA	NA	NA
cases	1143	58.4 (14.3)	20-91	33.3%	-10.50 (6.44)
controls 1	3120	58.5 (13.6)	20-90	61.7%	NA
controls 2	929	38.8 (11.8)	0-74	41.3%	NA
SORBS	621	NA	NA	NA	NA
cases	100	45.4 (6.6)	18-40	36.4%	NA
controls	521	28.3 (15.16)	18-80	45.0%	NA

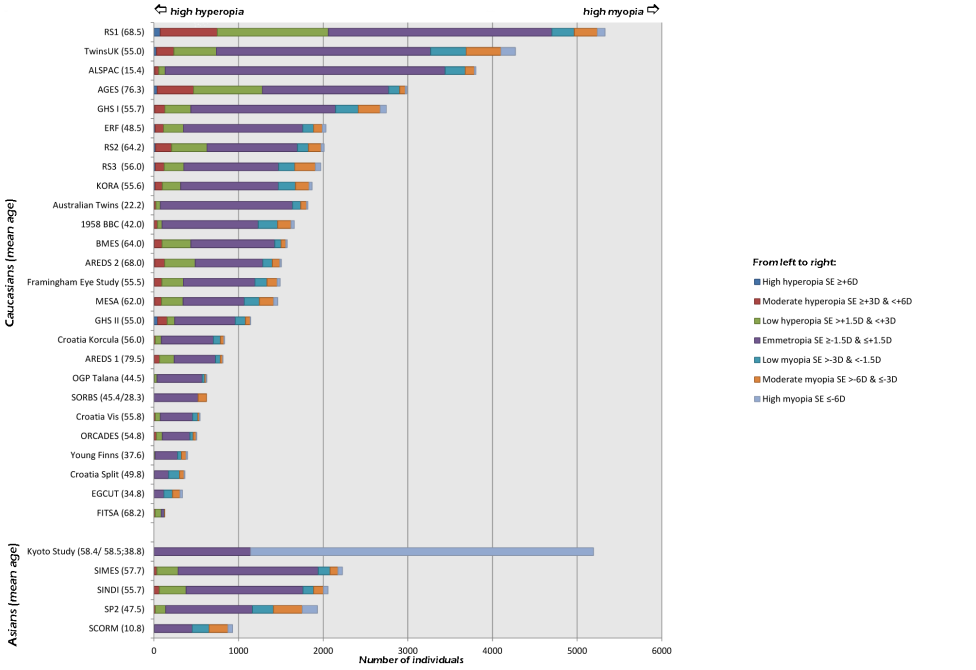


Figure 1. Mean age and distribution of spherical equivalent in all study cohorts

Table 2 shows the results of the meta-analysis of the 14 SNPs[20,25] at locus 15q14 and 5 SNPs at locus 15q25. The frequency of the effect allele C for top SNP rs634990 at locus 15q14 ranged from 0.38 to 0.64, while frequency of the effect allele A for top SNP rs939661 at 15q25 showed a larger variation, ranging from 0.28 to 0.63 (Supplementary Figure 1). The sample size of each SNP per study is provided in Supplementary Table 1. For locus 15q14, the magnitude and direction of the effects were consistent in all cohorts except Croatia Vis and SIMES. For locus 15q25, there was less consistency; for top SNP rs939661 8 cohorts - both Caucasian and Asian (Australian Twins, Croatia Split, Croatia Vis, EGCUT, FITSA, GHS II, ORCADES, and SIMES) - had a regression beta coefficient in the opposite direction to that of the other studies.

For locus 15q14, the replication set, consisting of all studies except the ones previously used in the discovery analysis, showed a statistically significant association between SE and all SNPs with a best *P*-value 4.53 x 10^{-14} for top SNP rs634990. Confirmation was achieved in 23 out of 25 Caucasian studies (overall *P* 3.87 x 10^{-12} for SNP rs634990), and in 3 out of 4 Asian studies (overall *P* 2.21 x 10^{-3} for SNP rs634990). Meta-analysis of the discovery and replication cohorts together provided *P*-value 9.20 x 10^{-23} for SNP rs634990.

For locus 15q25, neither Caucasian nor Asian validation studies replicated the original association. Meta-analysis of the combined set of the 5 SNPs yielded a lowest *P* 1.22 x 10^{-4} for SNP rs939661. As a subsequent analysis, we investigated locus 15q25 in more detail, and tested another 26 SNPs in 26 out of 29 cohorts (no data available in ALSPAC, AREDS 1, and EGCUT). This set of SNPs was not replicated either, however, meta-analysis including the discovery cohort was still

Table 2. Meta-analysis of allelic effects on spherical equivalent at locus 15q14 and 15q25

					Discovery (n=15,608)[1]			Replication (n=33,755)[2]			Caucasian (n=26,615)[3]			Asian (n=7,140)[4]			Meta-analysis (n=49,364)[5]		
SNP	Position	A1	A2	Freq	beta	se	*P*	beta	se	*P*	beta	se	*P*	beta	se	*P*	beta	se	*P*
locus 15q14																			
rs634990	32793365	C	T	0.49	-0.23	0.03	1.35E-14	-0.09	0.01	4.53E-14	-0.08	0.01	3.87E-12	-0.12	0.04	2.21E-03	-0.11	0.01	9.20E-23
rs560766	32788234	A	G	0.48	-0.20	0.03	4.82E-12	-0.09	0.01	3.53E-14	-0.08	0.01	3.91E-12	-0.12	0.04	1.47E-03	-0.10	0.01	1.03E-21
rs524952	32793178	A	T	0.48	-0.23	0.03	1.19E-14	-0.08	0.01	9.05E-13	-0.08	0.01	1.07E-11	-0.18	0.07	9.52E-03	-0.10	0.01	2.00E-21
rs688220	32786167	A	G	0.48	-0.20	0.03	4.43E-12	-0.08	0.01	1.01E-13	-0.08	0.01	1.38E-11	-0.12	0.04	9.80E-04	-0.10	0.01	3.44E-21
rs580839	32786121	A	G	0.48	-0.20	0.03	4.39E-12	-0.08	0.01	1.05E-13	-0.08	0.01	1.34E-11	-0.12	0.04	1.10E-03	-0.10	0.01	3.51E-21
rs11073060	32777143	A	C	0.48	-0.21	0.03	1.12E-12	-0.08	0.01	2.46E-13	-0.08	0.01	2.47E-11	-0.12	0.04	1.45E-03	-0.10	0.01	5.13E-21
rs4924134	32781857	G	A	0.45	-0.21	0.03	1.20E-12	-0.08	0.01	3.01E-13	-0.08	0.01	2.96E-11	-0.12	0.04	1.60E-03	-0.10	0.01	5.57E-21
rs7176510	32786771	T	C	0.45	-0.20	0.03	1.70E-11	-0.09	0.01	8.31E-14	-0.08	0.01	7.81E-12	-0.12	0.04	1.74E-03	-0.10	0.01	6.09E-21
rs619788	32782398	A	C	0.44	-0.20	0.03	3.94E-12	-0.08	0.01	2.21E-13	-0.08	0.01	2.29E-11	-0.12	0.04	1.54E-03	-0.10	0.01	6.97E-21
rs7163001	32777866	A	G	0.44	-0.21	0.03	1.26E-12	-0.08	0.01	6.28E-13	-0.08	0.01	4.16E-11	-0.11	0.04	2.81E-03	-0.10	0.01	1.41E-20
rs11073059	32776966	A	T	0.44	-0.21	0.03	1.98E-12	-0.08	0.01	8.78E-13	-0.08	0.01	4.85E-11	-0.11	0.04	3.64E-03	-0.10	0.01	2.63E-20
rs11073058	32776918	T	G	0.44	-0.20	0.03	2.23E-12	-0.08	0.01	8.52E-13	-0.08	0.01	4.84E-11	-0.11	0.04	3.50E-03	-0.10	0.01	2.68E-20
rs685352	32795627	G	A	0.46	-0.21	0.03	4.55E-13	-0.08	0.01	4.32E-12	-0.08	0.01	2.09E-10	-0.11	0.04	4.14E-03	-0.10	0.01	8.10E-20
rs8032019	32778782	G	A	0.40	-0.19	0.03	1.00E-10	-0.08	0.01	5.81E-12	-0.08	0.01	7.00E-10	-0.13	0.04	9.65E-04	-0.10	0.01	1.78E-18
locus 15q25					**Discovery (n=17,806)[1]**			**Replication (n=30,278)[2]**			**Caucasian (n=23,138)[3]**			**Asian (n=7,140)[4]**			**Meta-analysis (n=49,364)[5]**		
rs939661	77218118	A	G	0.51	-0.15	0.03	3.85E-09	-0.02	0.01	5.81E-02	-0.02	0.01	7.73E-02	-0.03	0.04	4.86E-01	-0.04	0.01	1.22E-04
rs939658	77238924	G	A	0.51	-0.15	0.03	1.85E-09	-0.02	0.01	1.60E-01	-0.02	0.01	2.16E-01	-0.04	0.05	3.94E-01	-0.04	0.01	4.32E-04
rs17175798	77251015	C	T	0.51	-0.15	0.03	1.99E-09	-0.02	0.01	1.81E-01	-0.01	0.01	2.38E-01	-0.05	0.06	3.70E-01	-0.04	0.01	6.12E-04
rs8033963	77242405	C	C	0.51	-0.15	0.03	1.86E-09	-0.01	0.01	2.18E-01	-0.02	0.01	2.20E-01	-0.01	0.04	8.42E-01	-0.04	0.01	9.37E-04
rs8027411	77248084	T	G	0.51	-0.15	0.03	2.07E-09	-0.01	0.01	2.49E-01	-0.02	0.01	2.16E-01	0.00	0.04	9.12E-01	-0.03	0.01	1.14E-03

1. for the 15q14 locus: RS-I, RS-II, RS-III, ERF, TwinsUK; for the 15q25 locus: TwinsUK, RS-I, RS-II, RS-III, ERF, 1958 Birth Cohort, Australian Twins (adult samples only); **2.** for the 15q14 locus: 1958 British Birth Cohort, AGES, ALSPAC, AREDS 1, AREDS 2, Australian Twins, BMES, Croatia Split, Croatia Vis, Croatia Korcula, EGCUT, FITSA, Framingham, GHS I, GHS II, KORA, MESA, ORCADES, OGP Talana, SCORM, SiMES, SINDI, SP2, Young Finns; for 15q25 locus: AGES, ALSPAC, AREDS 1, AREDS 2, BMES, Croatia Split, Croatia Vis, Croatia Korcula, EGCUT, FITSA, Framingham, GHS I, GHS II, KORA, MESA, ORCADES, OGP Talana, SCORM, SiMES, SINDI, SP2, Young Finns; **3.** for the 15q14 locus: 1958 British Birth Cohort, AGES, ALSPAC, AREDS 1, AREDS 2, Australian Twins, BMES, Croatia Split, Croatia Vis, Croatia Korcula, EGCUT, FITSA, Framingham, GHS I, GHS II, KORA, MESA, ORCADES, OGP Talana, Young Finns; for 15q25 locus: AGES, ALSPAC, AREDS 1, AREDS 2, BMES, Croatia Split, Croatia Vis, Croatia Korcula, EGCUT, FITSA, Framingham, GHS I, GHS II, KORA, MESA, ORCADES, OGP Talana, Young Finns; **4.** SP2, SIMES, SINDI, SCORM; **5**. all studies. Depicted in grey are significant Bonferroni corrected *P*-values of 3.57 x 10-3 for 15q14, and 1.0 x 10^{-2} for 15q25 (for GWAS data from discovery phase $P < 5 \times 10^{-8}$ was considered genome-wide significant). Freq, average frequency; A1, effect allele; A2, non-effect allele

significant (best *P* 2.07 x 10^{-4} for SNP rs1915726; Supplementary Table 3).

Meta-analysis of risk of myopia for top SNP

Genotype distributions for rs634990 at locus 15q14 were available for 28 out of 31 studies (all but FITSA, Australian Twins, and SORBS). There was no evidence of heterogeneity in the analyses of homozygote carriers (chi-squared 21.35 (d.f. 26), *P* 0.724, I^2 0.0%) or heterozygote carriers (chi-squared 24.22 (d.f. 26), *P* 0.564, I^2 0.0%). Therefore, only results from fixed effects meta-analysis were used. Figure 2 shows the forest plots for the risk of myopia for homozygous and heterozygous carriers of the top SNP rs634990. The OR of moderate to high myopia (SE ≤ -3 D) versus moderate to high hyperopia (SE ≥ +3 D) was 1.88 (95% CI 1.64, 2.16, P < 0.001) for homozygous carriers of the risk allele at the top SNP rs634990, and 1.33 (95% CI 1.19, 1.49, P < 0.001) for heterozygous carriers.

DISCUSSION

Chromosome 15q was first implicated in refractive error and myopia by genome-wide analysis of two large studies located in Northern Europe[20,25]. Here, in an international meta-analysis consisting of 31 independent studies from the CREAM consortium, we provide further support that the association with locus 15q14 is robust and present in both Caucasians and Asians. We combined the results with those of the initial study into a powerful meta-analysis of highly associated SNPs with a total study population of 55,177 participants. The combined results showed that all tested SNPs for locus 15q14 were associated with refractive errors, and that homozygous carriers of the top SNP rs634990 had approximately twice the risk of myopia. SNPs at the other locus, 15q25, could not be convincingly replicated.

This study has strengths and limitations. Major strengths of the study include the sample size and the inclusion of different ethnicities. The CREAM consortium represents the largest study on refractive error known to date. Previous replication studies have not been large scaled and focused on populations of the same ancestry[27-29]. Another advantage of our study is the incorporation of clinical relevant endpoints such as high myopia and high hyperopia. Among the limitations are differences in designs and methods of the studies. (1) Population-based as well as case control studies were incorporated. However, the latter were only two (Kyoto Study and SORBS) and both had results within the same range as the population-based studies. (2) Different types of equipment and measurement methods were used to detect refractive error. These differences are generally subtle, and are not likely to cause false findings. (3) Various methods of genotyping and imputation were used, and genotyping was not complete in all studies. All SNPs at 15q14 had similar effect; thus, we do not think this has influenced these associations. SNPs at 15q25 showed larger variation, and the incomplete genotyping may have underpowered this analysis.

Earlier replication of the 15q14 locus was reported by Hayashi et al.[26] in a Japanese sample of high myopic probands and controls. In a comparison of 1125 high myopes (axial length >26.1 mm) versus 1295 controls, the risk of high myopia was increased for the carriers of the initial top SNP rs634990 (OR 1.84 in homozygotes (95% CI 1.44-2.36)). Taken together with the current findings, this suggests that 15q14 plays a role in both common and high myopia.

A

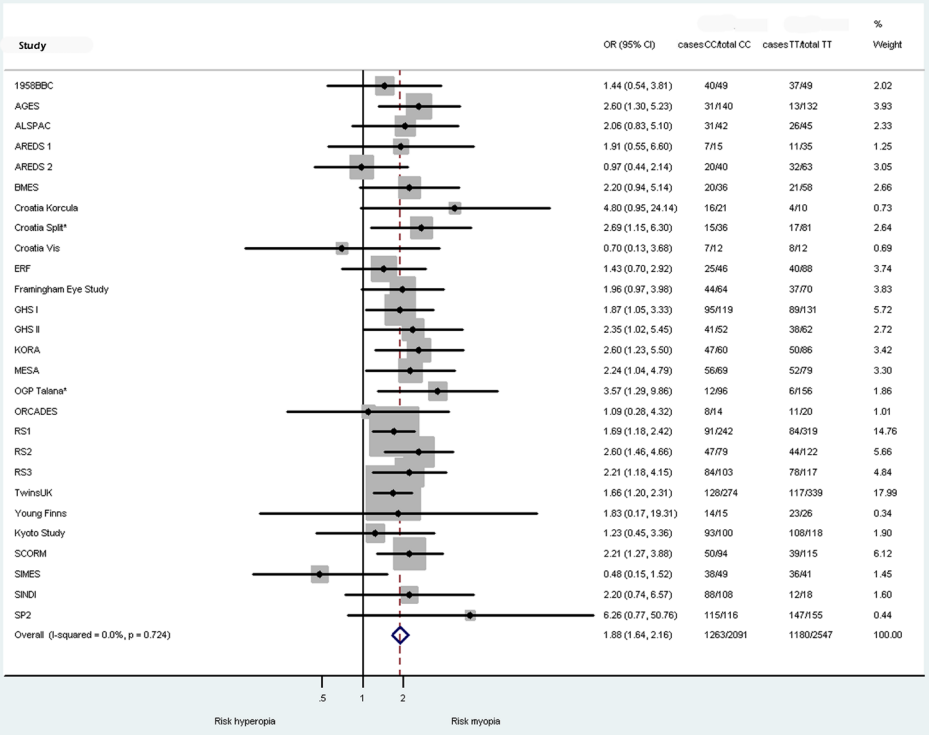
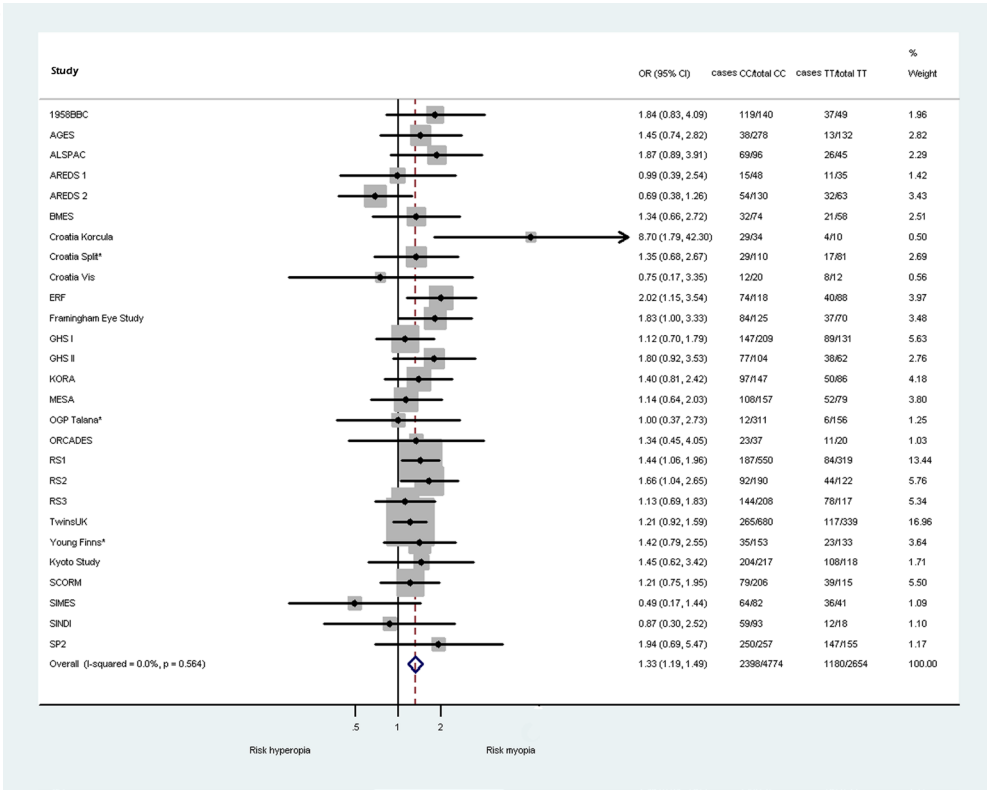


Figure 2. Forest plots of odds ratios of myopia (spherical equivalent ≤ –3 diopters) versus hyperopia (spherical equivalent ≥ +3 diopters) for top SNP rs634990.
Figure 2A. Homozygotes carriers of alleles TT vs CC for SNP rs634990. Figure 2B. Heterozygotes carriers of alleles TT vs TC for SNP rs634990. For studies without subjects with high or moderate hyperopia , emmetropia was used as a reference group.

The 15q14 associated region contains two interesting genes that are both well expressed in the retina, *GJD2* and *ACTC1. GJD2* encodes the Connexin36 protein, which plays a crucial role in the transmission and processing of visual signals in the retina by enabling intercellular transport of small molecules and ions in photoreceptors, amacrine and bipolar cells[30-33]. We speculated that the protein encoded by the other candidate gene, *ACTC1,* could play a role in scleral remodeling, given the fact that similar actin proteins have been shown to be increased in developing myopic tree shrew eyes[34]. Previous *GJD2*[25] and *ACTC1* (unpublished data) direct sequencing experiments did not reveal a functional variant, but the 15q14 locus appeared to harbor regulatory elements which may influence transcription of these genes[25].

The 15q25 region contains the interesting candidate gene *RASGRF1*, which is highly expressed in the retina and has previously been implicated in photoreception and visual sensory processes[35,36]. The association with this locus and gene is not robust, since none of the initial SNPs replicated significantly, and determination of more SNPs did not increase significance. A type 1 error may explain the initial finding. Another potential cause for the non-replication is a large variation in

B



allele frequencies. The range of allele frequencies at 15q25 (0.28 to 0.63) was only slightly larger than at 15q14 (0.38 to 0.64) in our consortium, making this an unlikely explanation (Supplementary Figure 1). Finally, population stratification within cohorts did not appear to play a major role, since only two cohorts had significant principal components, which were addressed in the analyses.

Other GWAS loci were only found for high myopia in Asian case control studies, and they were located on chromosomes 11q24.1[23], 5p15[21], 4q25[22], and 13q12.12[24]. The locus on chromosome 5p15 harbors the excellent candidate gene *CTNND2* which is involved in retinal morphogenesis, adhesion, retinal cell architecture integrity[37,38], and was replicated in subjects of the same ethnicity[28]. Replication studies for the 4q25[27] and 11q24.1[29] loci were only successful in case of the 4q25 locus; these loci did not have prominent candidate genes.

What should be the next steps? For 15q14, comprehensive resequencing of the entire associated region and the flanking genes can reveal the responsible gene defects which determine the association. Novel techniques such as next-generation sequencing are promising in this regard. Functional studies in knockout animals will shed light on potential protein effects. Lastly, evaluation of gene-environment interactions may explain phenotypic variation and help identify high risk groups. For myopia genetics in general, performance of a genome-wide meta-analysis is a logical next step. The current CREAM collaboration is an excellent platform for this project.

In summary, we have convincingly demonstrated that common variants at chromosome 15q14 influence susceptibility for myopia in both Caucasian and Asian populations around the world. Identification of functional variants and responsible genes that explain this association will provide more insight in the complex etiology of myopia.

MATERIALS AND METHODS

Subjects and phenotyping

A total of 31 study cohorts from the Consortium of Refractive Error and Myopia (CREAM) participated in this meta-analysis. 29 Population-based as well as 2 case-control studies were included. General methods, descriptives and phenotyping and genotyping methods of the study cohorts can be found in Table 1, the Supplementary Material and Supplementary Table 1, respectively. In short, 22 cohorts consisted of Caucasian, and 5 of Asian study subjects. All studies were performed with the approval of their local Medical Ethics Committee, and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

All studies used a similar protocol for phenotyping. Exclusion criteria were age ≤10 years, and bilateral cataract surgery, laser refractive procedures or other intra-ocular procedures which might alter refraction. Eligible participants underwent a complete ophthalmologic examination including a non-dilated measurement of refractive error (Table 1) of both eyes. Spherical equivalent was calculated according to the standard formula (SE=sphere + ½ cylinder), and the mean of two eyes was used for analysis. When data from only one eye were available, the SE of this eye was used. SE was categorized into low (SE from −1.5 to −3 D), moderate (SE from −3 to −6 D) and high (SE of −6 D or lower) myopia; and also into low (SE from +1.5 to +3 D), moderate (SE from +3 to +6 D) and high (SE of +6 D or higher) hyperopia. Emmetropia was defined as SE equal to or between −1.5 to +1.5 D.

Genotyping & imputation

DNA was extracted according to standard procedures, and genotyping and imputation of SNPs across the entire genome was performed using various methods (Table 1). Samples with a low call rate, with excess autosomal heterozygosity, with sex-mismatch, or outliers identified by the identity-by-state clustering analysis were excluded.

Statistical analysis

Meta-analysis of allelic effects on spherical equivalent

We selected 19 SNPs within loci 15q14 (14 SNPs) and 15q25 (5 SNPs) with a *P*-value of $< 10^{-6}$ from two previous GWAS[20,25]. Linear regression models with a 1 degree of freedom trend test were used to examine associations with SE as a quantitative trait outcome, adjusting for age and gender and significant principal components if applicable. From all population-based cohorts, we obtained effect allele, non effect allele, regression coefficient beta, standard error, *P* value, minor allele and minor allele frequency for each of these SNPs. METAL for Linux was used to perform a meta-analysis on betas and standard errors for all SNPs. First, discovery cohorts[20,25] and replication studies were analyzed separately, followed by a combined meta-analysis. As a second analysis,

26 additional SNPs within the same linkage disequilibrium (LD) block were selected and tested for association using the procedures mentioned above. For these analyses, Bonferroni corrected *P*-values (0.05 / number of tested SNPs) of 3.57×10^{-3} for 15q14, and 1.0×10^{-2} (5 SNPs, Table 2) or 1.92×10^{-3} (26 SNPs, Table 3 Supplementary Material) for 15q25 were considered statistically significant.

Meta-analysis of risk of myopia for top SNP

From all population-based and case control studies, we obtained genotype distributions of the replicated top SNPs. We calculated heterogeneity (chi-square, I^2 calculated and corresponding *P*-values) between studies, crude OR with corresponding 95% CI and *P*-value of moderate and high myopia versus moderate and high hyperopia with a random as well as fixed effects meta-analysis using Stata 11. When these analyses provided similar outcomes, data from fixed effect analysis were used. For studies without subjects with high or moderate hyperopia, emmetropia was used as a reference group. A standard *P*-value of <0.05 was considered statistically significant.

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3.3

Genome-wide meta-analyses of multi-ethnic cohorts identify multiple new susceptibility loci for refractive error and myopia

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ABSTRACT

Refractive error is the most common eye disorder worldwide, and a prominent cause of blindness. Myopia affects over 30% of Western populations, and up to 80% of Asians. The CREAM consortium conducted genome-wide meta-analyses including 37,382 individuals from 27 studies of European ancestry, and 8,376 from 5 Asian cohorts. We identified 16 new loci for refractive error in subjects of European ancestry, of which 8 were shared with Asians. Combined analysis revealed 8 additional loci. The new loci include genes with functions in neurotransmission (*GRIA4*), ion channels (*KCNQ5*), retinoic acid metabolism (*RDH5*), extracellular matrix remodeling (*LAMA2, BMP2)*, and eye development (*SIX6, PRSS56*). We also confirmed previously reported associations with *GJD2* and *RASGRF1*. Risk score analysis using associated SNPs showed a tenfold increased risk of myopia for subjects with the highest genetic load. Our results, accumulated across independent multi-ethnic studies, considerably advance understanding of mechanisms involved in refractive error and myopia.

INTRODUCTION & RESULTS

Refractive error is the most important cause of visual impairment in the world[1]. Myopia, or nearsightedness, in particular is associated with structural changes of the eye, increasing the risk of severe complications such as macular degeneration, retinal detachment, and glaucoma. The prevalence of myopia has been rising dramatically over the past few decades[2], and it is estimated that 2.5 billion people will be affected by myopia within a decade[3]. Although several genetic loci influencing refractive error have been identified[4-10], their contribution to phenotypic variance is small, and many more loci are expected to explain its genetic architecture.

Here the Consortium for Refractive Error and Myopia (CREAM) presents results from the largest international genome-wide meta-analysis on refractive error with data from 32 studies from Europe, the United States, Australia, and Asia. The meta-analysis was performed in three stages: as a first step, we investigated genome-wide association study (GWAS) results of 37,382 individuals from 27 populations of European ancestry (Supplementary Note, Supplementary Table 1) using spherical equivalent as a continuous outcome; as a second step, we aimed to test cross-ethnic transferability of the statistical significant associations from the first stage in 8,376 individuals from 5 Asian cohorts (Supplementary Note, Supplementary Table 1). As a third step, we performed a GWAS meta-analysis on the combined populations (total n = 45,758). Subsequently, we examined the influence of associated alleles on the risk of myopia in a genetic risk score analysis, and lastly, we evaluated gene expression in ocular tissues and explored potential mechanisms by which newly found loci may exert their effect on refractive development.

At step 1, we analyzed ~2.5 million autosomal single nucleotide polymorphisms (SNPs) which were obtained through whole-genome imputation of genotypes to HapMap 2. The inflation factors (λ_{GC}) of the test statistics in individual studies contributing to the meta-analysis ranged between 0.992 and 1.050, indicating excellent within-study control of population substructure (Supplementary Table 2). The overall lambda was 1.09, consistent with a polygenic inheritance model for refractive error (QQ plot, Supplementary Figure S1). We did not perform a lambda correction as Yang et al. have shown that in this situation substantial genomic inflation can be expected, even in the absence of population structure and technical artifacts[11]. We identified 309 SNPs that exceeding the conventional genome-wide significance threshold of P=5.0x10^{-8} in the European ancestry sample. These SNPs were clustered in 18 distinct genomic regions across 14 chromosomes (Figure 1, Table 1). At step 2, we investigated the 18 best associated SNPs in the Asian population: ten showed evidence of association (Table 1). The most significant association in both ancestry groups was at a previously identified locus on chromosome 15q14 in the proximity of the *GJD2* gene (SNP rs524952; $P_{combined}$=1.44x10^{-15})[4,12]. The locus near the *RASGRF1* gene was also replicated in the meta-analysis (SNP rs4778879; $P_{combined=}$4.25x10^{-11})[9], the remaining 16 genome-wide significantly associated loci had not previously been reported in association with refractive error. Those loci that were not significant in the smaller sized Asian population mostly had a similar effect size and direction of effect as in the European ancestry sample. At step 3, we identified 8 additional loci which exceeded genome-wide significance in the combined analysis (Table 2). Regional and forest plots of the associated loci are provided in Supplementary Figures 2 and 3.

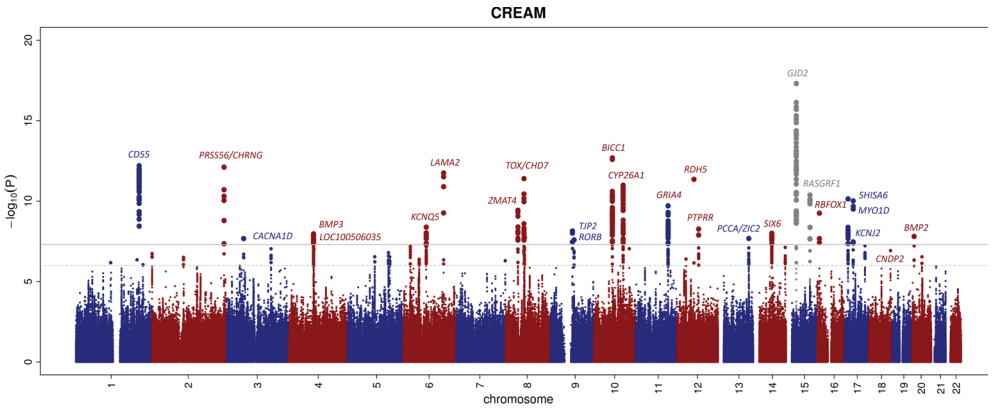

Figure 1. Manhattan plot of the GWAS meta-analysis for refractive error in the combined analysis (n = 45,758)
The plot shows –log10-transformed *P* values for all SNPs; the upper horizontal line represents the genome-wide significance threshold of $P < 5.0 \times 10^{-8}$; the lower line indicates *P* value of 10^{-6}. Previously reported genes are depicted in grey. The *A2BP1* gene is also known as *RBFOX1*.

Genotype distributions of the risk alleles were evaluated in Rotterdam Studies I-III (n=9,307). The clinical utility for the prediction of risk of myopia was evaluated by a weighted genetic risk score analysis based on the aggregate of effects (regression coefficients betas) of individual SNPs derived from the meta-analysis, using the middle risk category as a reference. Risk scores ranged from a mean risk score of 1.88 (95% CI 1.86 - 1.89) in the lowest risk score category to 3.63 (95% CI 3.61-3.65) in the highest risk score category. Having the lowest or the highest genetic risk score was associated with an odds ratio of 0.38 (95% CI 0.18-0.77), and an odds ratio of 10.97 (95% CI 3.727-31.251) of myopia, respectively (Figure 2). The predictive value (area under receiver operating characteristic curve, AUC) of myopia versus hyperopia was 0.67 (95% CI 0.65-0.69), a relatively high value for genetic factors in a complex trait[13,14]. The genetic variants explain 3.4% of the phenotypic variation in refractive error in the Rotterdam Study.

We examined the expression of genes harboring a genetic association signal by measuring levels of RNA in various eye tissues, and found most of these genes expressed in the eye (Supplementary Table 3). The genes *PRSS56, LOC100506035,* and *SHISA6* were not available in the expression data set; all other genes were expressed in the retina. Subsequently, we assessed the areas where our SNP hits reside for H3K27ac modification marks[15], and HaploReg[16] annotations for marks of active regulatory elements (Supplementary Table 4, Supplementary Figure 4). We found that many hits contain these elements, and alteration of regulatory function is therefore a suggestive mechanism.

The widely-accepted model for myopia development is that eye growth is triggered by a visually-evoked signaling cascade, which originates from the sensory retina, traverses the retinal pigment epithelium and choroid, and terminates in the sclera, where active extracellular matrix (ECM) remodeling results in a relative elongation of the eye[17]. Many of the genes in or near the identified loci can be linked to biological processes that drive this cascade. Neurotransmission in the retina is a necessary mechanism for eye growth regulation; the most significantly associated gene *GJD2* plays a role herein. This gene forms a gap junction between neuronal cells in the retina, enabling

Table 1. Genome-wide significant hits for refractive error in the European ancestry population with results in Asian population and combined analysis
Summary of SNPs that showed genome-wide significant (P<5×10-8) association with spherical equivalent (SE) in subjects of European ancestry (stage 1), with results of replication in Asians (stage 2) and combined analysis (stage 3). Previously reported genes are marked in grey. We tested for heterogeneous effects between the Asian and the European ancestry sample, for which P values are shown.

Locus #	SNP	Chr	Position	Gene	A1/A2	MAF	Beta	SEM	P-value	MAF	Beta	SEM	P-value	Beta	SEM	P-value	P-value
						Stage 1 (n = 37,382)				*Stage 2 (n = 8,376)*				*Combined (n = 45,758)*			*Het*
1	rs1652333	1	203858855	CD55	G/A	0.32	-0.115	0.018	6.29E-11	0.42	-0.099	0.035	5.00E-03	-0.112	0.016	3.05E-12	0.94
2	rs1656404	2	233205446	PRSS56	A/G	0.21	-0.151	0.025	2.38E-09	0.11	-0.167	0.069	1.60E-02	-0.153	0.024	7.86E-11	0.83
	rs1881492	2	233406997	CHRNG	T/G	0.22	-0.145	0.022	1.28E-10	0.15	-0.057	0.110	6.09E-01	-0.139	0.021	5.15E-11	0.88
3	rs14165	3	53847407	CACNA1D	A/G	0.32	0.095	0.017	4.36E-08	0.12	0.120	0.100	2.29E-01	0.096	0.017	2.14E-08	0.25
4	rs1960445	4	81930813	BMP3	C/T	0.17	-0.147	0.026	1.19E-08	0.11	0.034	0.055	5.32E-01	-0.114	0.024	1.25E-06	0.31
5	rs12205363	6	129834628	LAMA2	C/T	0.10	0.228	0.034	1.13E-11	0.02	0.553	0.236	1.92E-02	0.235	0.033	1.79E-12	0.93
6	rs4237036	8	61701056	CHD7	C/T	0.35	0.097	0.017	1.52E-08	0.23	0.043	0.040	2.81E-01	0.089	0.016	1.82E-08	0.76
	rs7837791	8	60179085	TOX	T/G	0.49	0.106	0.017	9.22E-10	0.39	0.103	0.035	4.00E-03	0.106	0.015	3.99E-12	0.70
7	rs7829127	8	40726393	ZMAT4	G/A	0.25	0.116	0.020	3.04E-09	0.11	0.112	0.055	4.23E-02	0.116	0.018	3.69E-10	0.66
8	rs7042950	9	77149836	RORB	G/A	0.24	-0.113	0.020	1.02E-08	0.42	-0.040	0.037	2.72E-01	-0.096	0.018	4.15E-08	0.83
9	rs10882165	10	94924323	CYP26A1	T/A	0.42	-0.111	0.016	1.25E-11	0.20	-0.060	0.056	2.84E-01	-0.107	0.016	1.03E-11	0.90
10	rs7084402	10	60265403	BICC1	G/A	0.48	-0.111	0.016	7.23E-12	0.50	-0.094	0.035	7.34E-03	-0.108	0.015	2.06E-13	0.71
11	rs11601239	11	105061808	GRIA4	C/G	0.46	-0.092	0.017	3.45E-08	0.42	-0.129	0.058	2.70E-02	-0.095	0.016	5.92E-09	0.83
12	rs3138144	12	56114768	RDH5	C/G	0.48	0.113	0.018	4.28E-10	0.45	0.157	0.072	3.00E-02	0.119	0.017	4.44E-12	0.09
13	rs2184971	13	100818091	PCCA	G/A	0.44	0.095	0.016	5.90E-09	0.22	0.022	0.040	5.84E-01	0.085	0.015	2.11E-08	0.96
	rs8000973	13	100691366	ZIC2	T/C	0.47	0.089	0.016	4.24E-08	0.22	0.030	0.041	4.63E-01	0.081	0.015	5.10E-08	0.50
14	rs524952	15	35005885	GJD2	A/T	0.48	-0.154	0.021	1.11E-13	0.44	-0.193	0.060	1.00E-03	-0.158	0.020	1.44E-15	0.22
15	rs4778879	15	79372874	RASGRF1	G/A	0.44	-0.103	0.017	1.27E-09	0.39	-0.103	0.043	1.50E-02	-0.102	0.015	4.25E-11	0.15
16	rs17183295	17	31078271	MYO1D	T/C	0.23	-0.132	0.021	3.04E-10	0.16	-0.166	0.144	2.49E-01	-0.131	0.020	9.66E-11	0.34
17	rs4793501	17	68718733	KCNJ2	C/T	0.42	0.096	0.016	3.21E-09	0.44	0.010	0.034	7.64E-01	0.080	0.014	2.79E-08	0.04
18	rs12971120	18	72174022	CNDP2	G/A	0.23	0.108	0.020	4.39E-08	0.30	0.014	0.063	8.27E-01	0.099	0.019	1.85E-07	0.49

SNP, single nucleotide polymorphism; Chr, chromosome; Gene, nearest gene according to reference NCBI build 37; A1, reference allele; A2, other allele, MA, Minor Allele; MAF, average Minor Allele Frequency; Beta, effect size on spherical equivalent in diopters based on allele A1; SEM, standard error of the mean; Het, P value for heterogeneity.

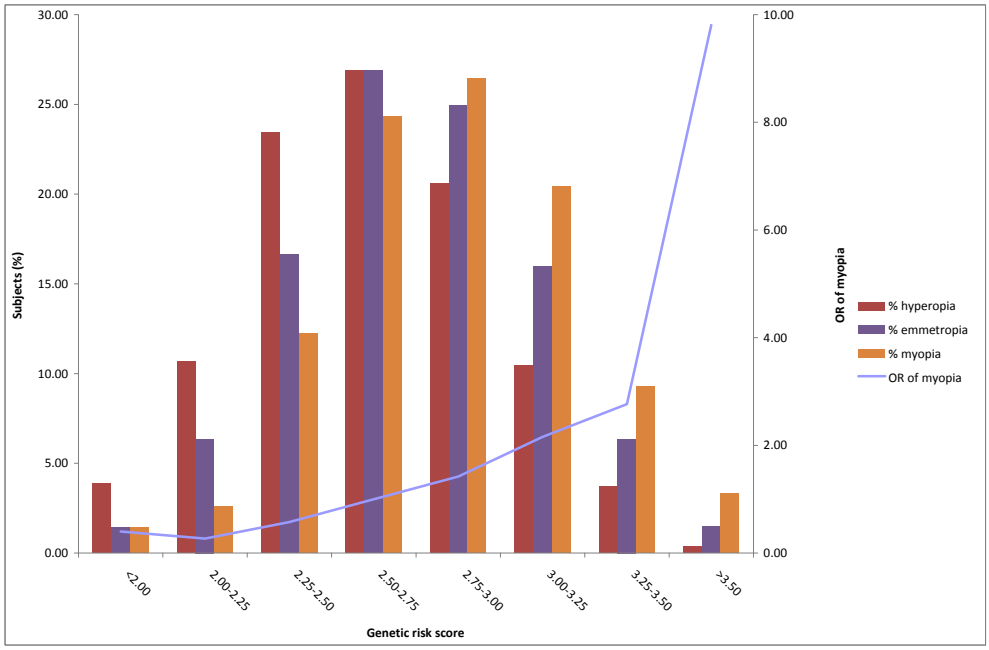

Figure 2. Genetic risk score for myopia

Distribution of subjects from Rotterdam Study I-III (n= 9,307) with myopia (SE ≤ -3 D), emmetropia (SE ≥ -1.5 D & ≤ 1.5 D) and hyperopia (SE ≥ 3 D) as a function of genetic risk score. This score is based on the regression coefficients and allele dosages of the associated SNPs for all 26 loci identified in the meta-analysis. The mean OR of myopia was calculated per risk category, using the middle risk score category (risk score 2.50; 2.75) as a reference.

intracellular exchange of small molecules and ions. The other previously-reported gene *RASGRF1* is a nuclear exchange factor that promotes GDP/GTP exchange on Ras-family GTPases, and is involved in synaptic transmission of photoreceptor responses[18,19]. Both *GJD2* and *RASGRF1* knockout mice show retinal photoreception defects[18,20]. One of the newly identified genes, *GRIA4* (SNP rs11601239; $P_{combined}$=5.92x10^{-9}) also has a potential function in this pathway. This gene is a glutamate-gated ion channel that mediates fast synaptic excitatory neurotransmission[21], is present in various retinal cells[22], has been shown to be critical for light signaling in the retina[23] and emmetropization[24]. Another gene involved in synaptic transmission is *A2BP1* (also known as *RBFOX1*; SNP rs17648524; $P_{combined}$=5.64x10^{-10}), an RNA-binding splicing regulator which modulates membrane excitability[25].

We identified for the first time a number of genes involved in ion transport, channel activity and maintenance of membrane potential. *KCNQ5*, a potassium channel regulator (SNP rs7744813; $P_{combined}$=4.18x10^{-9}), participates in the transport of K^+ from the retina to the choroid, and may contribute to voltage-gated K^+ channels in photoreceptors and retinal neurons associated with myopia[26,27]. *CD55* (SNP rs1652333; $P_{combined}$=3.05x10^{-12}) is known to elevate cytosolic calcium ion concentration. Other ion channel genes include *CACNA1D*, a voltage-sensitive calcium channel regulator; *KCNJ2* , a regulator of potassium ion transport; *CHRNG*, a nicotinic cholinergic receptor; and *MYO1D,* a putative binder of calmodulin, which mediates Ca^+ sensitivity to KCNQ5 ion channels.

Table 2. Additional genome-wide significant hits from the combined meta-analysis

Summary of SNPs that showed genome-wide significant (P<5×10-8) association with spherical equivalent (SE) in the combined analysis (stage 3), with results in subjects of European ancestry (stage 1) and Asians (stage 2). We tested for heterogeneous effects between the two ancestries, for which P values are shown.

Locus #	SNP	Chr	Position	Gene	A1/A2	Beta	SEM	P-value	MAF	Beta	SEM	P-value	MAF	Beta	SEM	P-value	P-value
						Combined (n = 45,758)			*Stage 1 (n = 37,382)*				*Stage 2 (n = 8,376)*				*Het*
1	rs9307551	4	80530670	*LOC100506035*	A/C	-0.099	0.017	1.09E-08	0.25	-0.097	0.020	1.37E-06	0.50	-0.105	0.035	3.06E-03	0.70
2	rs7744813	6	73643288	*KCNQ5*	C/A	0.112	0.019	4.18E-09	0.41	0.114	0.021	6.80E-08	0.33	0.094	0.046	4.30E-02	0.14
3	rs11145465	9	71766592	*TJP2*	A/C	-0.124	0.021	7.26E-09	0.25	-0.125	0.023	6.92E-08	0.07	-0.136	0.091	1.35E-01	0.14
4	rs12229663	12	71249995	*PTPRR*	G/A	0.099	0.017	5.47E-09	0.27	0.104	0.019	5.46E-08	0.36	0.080	0.052	1.23E-01	0.74
5	rs1254319	14	60903756	*SIX6*	A/G	-0.088	0.015	1.00E-08	0.32	-0.088	0.017	2.03E-07	0.34	-0.087	0.036	1.57E-02	0.59
6	rs17648524	16	7459682	*A2BP1*	C/G	-0.118	0.019	5.64E-10	0.36	-0.116	0.022	7.48E-08	0.14	-0.140	0.058	1.60E-02	0.24
7	rs2969180	17	11407900	*SHISA6*	A/G	-0.101	0.015	7.29E-11	0.36	-0.101	0.019	7.51E-08	0.45	-0.097	0.034	4.00E-03	0.41
8	rs235770	20	6761764	*BMP2*	T/C	-0.089	0.016	1.57E-08	0.39	-0.088	0.017	1.34E-07	0.33	-0.087	0.050	8.20E-02	0.78

SNP, single nucleotide polymorphism; Chr, chromosome; Gene, nearest gene according to reference NCBI build 37; A1, reference allele; A2, other allele, MA, Minor Allele; MAF, average Minor Allele Frequency; Beta, effect size on spherical equivalent in diopters based on allele A1; SEM, standard error of the mean; Het, P value for heterogeneity. The *A2BP1* gene is also known as *RBFOX1*.

Retinoic acid is synthesized in the retina and highly expressed in the choroid and has been implicated in eye growth in experimental myopia models[28-30]. Retinol dehydrogenase 5 (*RDH5),* a novel refractive error susceptibility gene (SNP rs3138144; $P_{combined}$=4.44x10^{-12}), is involved in the recycling of 11-cis-retinal in the visual cycle[31]. Mutations in *RDH5* cause congenital stationary night blindness (OMIM #136880), a disease associated with myopia. Other genes involved in retinoic acid metabolism are *RORB* (RAR-related orphan receptor), and *CYP26A1*, genes that were significant in the European ancestry studies. Notably, retinoic acid contributes to ECM remodeling by regulating cell differentiation.

ECM remodeling of the sclera is the pathological hallmark in myopia development. *LAMA2* (*laminin α2*, SNP rs12205363; $P_{combined}$=1.79x10^{-12}) is the most prominent gene in this respect. *LAMA2* forms a subunit of the heterotrimer laminins which are essential components of basement membranes, stabilizing cellular structures and facilitating cell migration[32]. The two bone morphogenic genes (*BMP2,* SNP rs235770; $P_{combined}$=1.57x10^{-8}*; BMP3*) can also be placed within the ECM architecture. They are members of the transforming growth factor-β (TGFβ) super-family, regulate growth and differentiation of mesenchymal cells, and may orchestrate the organization of other connective tissues than bone such as sclera. Remarkably, *BMP2* shows bidirectional expression in retinal pigment epithelium in myopia animal models[33].

Genes involved in eye development appeared as a separate entity among the gene functions. *SIX6* (SNP rs1254319; $P_{combined}$=1.00x10^{-8}*)* has been linked to anophthalmia and glaucoma[34,35], *PRSS56 (protease serine 56,* SNP rs1656404; $P_{combined}$=7.86x10^{-11}*)* to microphthalmia[36-38], *CHD7* to CHARGE syndrome, a congenital condition with severe eye structural defects, and *ZIC2* to brain development including visual perception. For the remaining novel gene associations, a mechanism in the pathogenesis of myopia is not immediately clear. Results from Ingenuity and the Protein Link Evaluator[39] (Supplementary Figure 5) visualize the subcellular location of all associated genes, and illustrates their interrelationships. Direct connections between genes were surprisingly infrequent, suggesting molecular disease heterogeneity or functional redundancy in the pathobiological events involved in development of refractive error and myopia.

In summary, we identified 24 new chromosomal loci associated with refractive error through a large-scale meta-analysis of GWAS from international multi-ethnic studies. The significant overlap in genetic loci for refractive error between subjects of European ancestry and Asians provides evidence for shared genetic risk factors between the populations. The tenfold increased risk of myopia for those carrying the highest number of risk alleles depicts the clinical significance of our findings. Further elucidation of the mechanisms by which these loci affect eye growth carries the potential to improve the visual outcome of this common trait.

METHODS

Study design

We performed a meta-analysis on directly genotyped and imputed SNPs from individuals of European ancestry in 27 studies, with a total of 37,382 individuals. Subsequently, we evaluated significant SNPs in 8,376 subjects of Asian origin from 5 different studies, and performed a meta-analysis on all studies combined .

Subjects and phenotyping

All studies participating in this meta-analysis are part of the Consortium for Refractive Error and Myopia (CREAM). All studies had a population-based design and had a similar protocol for phenotyping (Supplementary Table 1). Eligible participants underwent a complete ophthalmologic examination including a non-dilated measurement of refractive error of both eyes. Exclusion criteria were all conditions that could alter refraction, such as cataract surgery, laser refractive procedures, retinal detachment surgery, keratoconus, or ocular or systemic syndromes. Inclusion criteria were persons aged 25 years and over who had data on refractive error and genotype.

The meta-analysis of step 1 was based on 27 studies of European ancestry: 1958 British Birth Cohort, ALSPAC, ANZRAG, AREDS1a1b, AREDS1c, CROATIA-Korcula, CROATIA-Split, CROATIA-Vis, EGCUT, FECD, TEST/BATS, FITSA, Framingham, GHS 1, GHS 2, KORA, ORCADES, TwinsUK, WESDR, YFS, ERF, DCCT, BMES, RS-I, RS-II, RS-III, and OGP Talana. The second step was formed by 5 Asian studies: Beijing Eye Study, SCES, SIMES, SINDI, and SP2.

General methods, demographics and phenotyping and genotyping methods of the study cohorts can be found in the Supplementary Note and Supplementary Table 1. All studies were performed with the approval of their local Medical Ethics Committee, and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Genotyping and imputation

Particulars of genotyping in each cohort, particular platforms used to generate genotyping and methods of imputation can be found in more detail in the Supplementary Table 5. To produce consistent datasets and enable meta-analysis of studies across different genotyping platforms, the cohorts performed genomic imputation on the HapMap Phase 2 available genotypes with MACH[40] or IMPUTE[41], using the appropriate ancestry as templates.

Each cohort applied stringent quality control procedures prior to the imputation, including minor allele frequency cutoffs, Hardy-Weinberg equilibrium ($P > 10^{-7}$), genotypic success rate (>95%), Mendelian inconsistencies, exclusion of individuals with more than 5% shared ancestry (exception made for family-based cohorts in which due adjustment for family relationship was made) and removal of all individuals whose ancestry as determined through genetic analysis did not match the prevailing ancestry group of the own cohort. SNPs with low imputation quality were filtered using metrics specific to the imputation method and thresholds used in their previous GWAS analyses. Hence, imputation quality criteria varied slightly among studies, and low-confidence imputed SNPs were omitted in the meta-analysis for individual studies.

Statistical analysis

Spherical equivalent was calculated according to the standard formula (SE=sphere + ½ cylinder), and the mean of two eyes was used for analysis. When data from only one eye was available, the SE of this eye was used.

Each cohort performed association analyses in which the spherical equivalent (determined as described above) was the dependent variable and genotypes (number of alleles in each of the

HapMap2 loci) as the independent variables. Analyses in all cases also adjusted for sex and age at the time of phenotype measurement. In family-based cohorts score-test based association test was used to adjust for within-family relatedness (see Supplementary Note)[42,43]. Study-specific lambda estimates are shown in Supplementary Table 2.

All study effect estimates were corrected using genomic control and were oriented to the positive strand of the NCBI build 36 reference sequence of the human genome, which was the genomic build on which most available genotyping platforms were based. The coordinates and further annotations of the SNPs were further converted into build 37, the most recent of the available builds at the time of writing.

Meta-analyses used effect size estimations (beta regression coefficients) and standard errors from individual cohorts' summary statistics. Random-effects were assumed for all the meta-analyses which were performed using GWAMA[44]. We tested for heterogeneous effects between the two ancestries using METAL[45] for Linux. For the purpose of these analyses, we defined significance as equal to or better than the conventional multiple testing genome-wide thresholds of association ($P<5.0 \times 10^{-8}$) for stage 1 and nominally significant probabilities ($P<0.05$) for stage 2. Manhattan, regional plots and forest plots were made using R and Locuszoom[46].

For the Rotterdam Study I-III, a weighted genetic risk score per individual was calculated using the regression coefficients from the GWAS meta-analysis model for the association of SNPs within the associated 26 loci (Table 1, Table 2; per locus only one SNP was included in the analysis) and the individual allele dosages per genotype to evaluate the relationships between myopia (SE ≤ - 3 D), emmetropia (-1.5 D ≤ SE ≤ 1.5 D) and hyperopia (SE ≥ +3 SD). The weighted risk scores were categorized and mean odds ratios per risk score category were calculated for subjects with myopia versus hyperopia, using the middle risk score category as a reference. Subsequently, the area under the receiver curve (AUC) was calculated for myopia versus emmetropia and myopia versus hyperopia. Lastly, the proportion of variance of spherical equivalent explained by the identified SNPs was calculated. For these analyses, we used SPSS version 20.0.0 (SPSS Inc.).

Gene expression data in human eye tissue

Independently designed, collected, and reported human ocular tissue array data from two different sources, as well as literature reviews were used to verify evidence of expression of the candidate genes.

RPE, photoreceptors and choroid

Human gene expression data of RPE, photoreceptors and choroid were obtained essentially as described[47] and the dataset has been deposited in NCBI's Gene Expression Omnibus[48] (GEO series accession number GSE20191). In short, postmortem eye bulbs (retinal pigment epithelium was obtained from six donor eyes, choroid was obtained from three donor eyes and photoreceptors were obtained from three donor eyes), provided by the Corneabank Amsterdam, were rapidly frozen using liquid nitrogen. Donors were between 63 and 78 years old and had no known history of eye pathology. Cryosections were cut from the macula, and histology was used to confirm a normal histological appearance. Retinal pigment epithelium, photoreceptor and choroidal cells

were isolated from macular sections using a Laser Microdissection System (PALM). Total RNA was isolated and the mRNA component was amplified, labeled and hybridized to a 44K microarray (Agilent Technologies)[49]. At least three to six microarrays were performed per tissue. Sample isolation, procedures and expression microarray analysis were carried out according to MIAMI guidelines. As a measure of the level of expression, we sorted all the genes represented on the 44K microarray by increasing their expression, and we calculated the corresponding percentiles (Supplementary Table 3a).

Sclera, cornea and optic nerve

We assessed expression of the associated genes in sclera, cornea and optic nerve tissue in an additional dataset (unpublished data). Adult eyes were obtained from the North Carolina Eye Bank (Winston-Salem, North Carolina). All whole globes were immersed in RNALater (Quiagen, Hilden, Germany) within 6.5 hours of collection, shipped overnight on ice, and dissected on the day of arrival. The retina, choroid and scleral tissues were isolated at the posterior pole using a circular, double embedded technique using round 7 mm and 5 mm biopsy punches. To reduce contamination of retina to the other ocular tissues samples, the second biopsy punch of 5 mm was used in the center of the 7 mm punch after retinal removal. RNA samples (quality control of RNA concentration and 260/280 nm ratios using Nanodrop®) (Invitrogen, Carlsbad, California, USA) were hybridized to whole genome microaray Illumina® HumanHT-12 v4 Expression BeadChips (over 25,000 genes and 48,000 probes) in two batches. The first batch was hybridized to adult RPE, choroid, and sclera RNA samples (n=6). The second batch of newer chips with additional probes was hybridized to adult optic nerve and cornea samples (n=6). The data were exported from Illumina® GenomeStudio and log2 transformed. Sample outliers were determined by principle component analyses using the Hoteling's T2 test[50] (at 95% confidence interval) and removed from further analyses. The data intensity was normalized by Quantile normalization followed by Multichip Averaging[51] to reduce chip effects. For each tissue type, the probes with signal intensities below background levels and those with the lowest (5%) signal intensities (detection $P<0.10$) were excluded. Evidence of expression in the remaining probes was defined by detection $P<0.05$. Probes with detection P values < 0.10 and > 0.05 required additional tissue expression support from EyeSAGE or literature reports (Supplementary Table 3b).

Search for regulatory elements

We used the 'Integrated Regulation from ENCODE' track in the UCSC genome browser to look at H3K27ac modificiation marks as a mark of active regulatory elements. Numbers of H3K27ac modification marks were counted between the associated topSNP from a locus and the nearest gene and within (the nearest) gene itself. We also used HaploReg[16] annotations to look for other signs of regulatory activity at the site of the associated SNP itself, such as enhancer histone marks, DNAse hypersensitivity sites, binding proteins and motifs changed.

Pathway analyses

We used two different programs for pathway analysis; Ingenuity, version August 2012, application build 172788, content version 14197757) and the Disease Association Protein-Protein Link Evaluator (DAPPLE)[39].

Subcellular localization assignment and functional annotation of myopia associated disease genes as well as molecular pathway analysis was carried out using the Ingenuity knowledge database. The candidate myopia disease genes discovered in this study were entered into the Ingenuity knowledge database (IPA). We used the "IPA toggle subcellular layout" function to show the subcellular location (extracellular, plasma membrane, cytoplasm, nucleus, unknown) of the proteins corresponding to these genes, which yield a first glance which signaling molecules and pathways are involved in myopia. Subsequently, we used the IPA "connect" function to discover potential direct or indirect functional relationships or molecular pathways in between these entries. This yielded surprisingly little hits, which suggest molecular disease heterogeneity and/or functional redundancy in the pathobiological events leading to myopia. Next, we used the IPA "overlay" function to annotate the myopia candidate disease genes with (their involvement in) "functions and diseases", "canonical pathways" and a range of custom made gene lists from previous studies, including photoreceptor, RPE, and choroidal specific transcripts (partly published[52]). Lastly, we used the Disease Association Protein-Protein Link Evaluator (DAPPLE)[39] to look for physical connections between proteins encoded from disease-genes associated regions.

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3.4

Genome-wide meta-analysis of myopia and myperopia provides evidence for replication of 11 loci

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ABSTRACT

Refractive error (RE) is a complex, multifactorial disorder characterized by a mismatch between the optical power of the eye and its axial length that causes object images to be focused off the retina. The two major subtypes of RE are myopia (nearsightedness) and hyperopia (farsightedness), which represent opposite ends of the distribution of the quantitative measure of spherical refraction. We performed a fixed effects meta-analysis of genome-wide association results of myopia and hyperopia from 9 studies of European-derived populations: AREDS, KORA, FES, OGP-Talana, MESA, RSI, RSII, RSIII and ERF. One genome-wide significant region was observed for myopia, corresponding to a previously identified myopia locus on 8q12 ($p=1.25x10^{-8}$), which has been reported by Kiefer et al as significantly associated with myopia age at onset and Verhoeven et al as significantly associated to mean spherical-equivalent (MSE) refractive error. We observed two genome-wide significant associations with hyperopia. These regions overlapped with loci on 15q14 (minimum p value=$9.11x10^{-11}$) and 8q12 (minimum p value $1.82x10^{-11}$) previously reported for MSE and myopia age at onset. We also used an intermarker linkage- disequilibrium-based method for calculating the effective number of tests in targeted regional replication analyses. We analyzed myopia (which represents the closest phenotype in our data to the one used by Kiefer et al) and showed replication of 10 additional loci associated with myopia previously reported by Kiefer et al. This is the first replication of these loci using myopia as the trait under analysis. “Replication-level” association was also seen between hyperopia and 12 of Kiefer et al’s published loci. For the loci that show evidence of association to both myopia and hyperopia, the estimated effect of the risk alleles were in opposite directions for the two traits. This suggests that these loci are important contributors to variation of refractive error across the distribution.

INTRODUCTION

Refractive errors (RE) are etiologically complex, multifactorial disorders characterized by a mismatch between the optical focal length of the eye and its axial length. This optical mismatch causes images to be focused away from the retina. The two major subtypes of spherical RE are myopia (nearsightedness) and hyperopia (farsightedness). Clinically significant myopia affects at least 25% of individuals over age 40 in the United States and western Europe, while hyperopia affects about 10% of individuals in this same age group[1]. Recent reports show that the prevalence of myopia has increased significantly in the United States over the last 3 decades; myopia of 2 (D) diopters or more was estimated to afflict 41.6% of Americans aged 12 to 54 years in 1999-2004, compared to only 25% in 1971-1972[2]. The myopia epidemic is most acute in East Asia, where prevalence estimates of myopia (of at least 0.5 D) routinely surpass 70% among late teenagers and young adults[3-5] A recent study of 19 year-old male military conscripts from Seoul, Korea, found that a staggering 96.5% were myopic[6].

The causes of RE are complex and are a combination of environmental and genetic factors[7]. Twin studies have reported a heritability greater than 0.50 for RE[8]. Several studies have calculated the heritability to be as high as 0.98 for myopia and 0.75 for hyperopia[9-12]. The search for environmental factors influencing RE have mostly focused on myopia. These include near work and time spent outdoors during childhood and teenage years[13-16]. Genome-wide association studies have become an essential tool in the study of traits such as RE, and to date there have been 67 published loci for refraction phenotypes[17]. In particular, Kiefer et al[18] performed a genome-wide association study of myopia using self-reported age at onset in 45,771 participants and found 22 significant genome-wide associations. Verhoeven et al performed a genome wide association of the quantitative trait mean spherical equivalent (MSE) and found 24 significant genome-wide associations (2 of which were replications of previously published loci)[19]. Thirteen loci were genome-wide significant in both the Kiefer et al and Verhoeven et al studies[20].

Here we present the results of a genome-wide association meta-analysis of 2 dichotomous RE traits, myopia and hyperopia (adjusted for age, sex and years of education), in 9 populations: the Age-Related Eye Disease Study (AREDS), the Cooperative Health Research in the Region of Augsburg (KORA) the Framingham Eye Study (FES), Ogliastra Genetic Park-Talana (OGP-Talana) Study, the Multi-ethnic Study of Atherosclerosis (MESA), the Rotterdam Eye Studies I, II and III (RSI, RSII, RSIII) and the Erasmus Rucphen Family Study (ERF). These are termed the discovery meta-analyses of myopia and hyperopia hereafter. Eight of the discovery samples were previously included in the meta-analysis of refractive error by Verhoeven et al[19]. One sample, the MESA study, was not included in either Kiefer et al[18] or Verhoeven et al's studies[19,21]. We attempted replication of significant and suggestive associations from the discovery meta-analyses through meta-analysis of association studies using these same trait definitions to these selected regions in 8 additional studies: the 1958 British Birth Cohort, the Blue Mountains Eye Study (BMES), the CROATIA-Vis Island Study, the CROATIA-Korcula Study, the Diabetes Control and Complications Trial (DCCT), the Orkney Complex Disease Study (ORCADES), the TwinsUK Study, and the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR). All of these studies

were previously included in the meta-analysis of refractive error by Verhoeven et al[19]. Finally, we examined the results of our discovery meta-analyses of myopia and hyperopia in the regions found to be associated with myopia age at onset by Kiefer et al[18]. In genetic association studies, the term replication is generally used to mean detection of statistical association of the same trait to the same associated genetic locus in an independent set of data. Here, we also use the term replication when discussing the results of our myopia trait (adjusted for age at examination, sex and years of education) since it is expected to be quite similar to the age at onset of myopia trait used by Kiefer et al. in their study[18]. We show independent replication of 11 of Kiefer et al's loci for myopia age at onset[18], and while our myopia trait is not exactly the same as that of Kiefer et al[18], it is the closest phenotype available in our data. We also examined these same regions for association to hyperopia. The association to hyperopia would not constitute a "replication" of Kiefer et al's myopia findings, but association with this related trait may help to clarify the complex genetic underpinnings of refractive error.

MATERIAL AND METHODS

Populations

The nine GWASs meta-analyzed in the discovery GWAS portion of this study included subjects aged 35-84 years from the Cooperative Health Research in the Region of Augsburg Study (KORA F3, Southern Germany), subjects aged 55-80 from the Age-related Eye Study (AREDS), unrelated subjects aged 28-84 from the Framingham Eye Study (FES), subjects aged 46-86 from the Multi-Ethnic Study of Atherosclerosis (MESA) study, and subjects aged 18-88 from the Ogliastra Genetic Park-Talana (OGP-Talana) study in Sardinia, subjects aged 55 and older from the Rotterdam Eye Study I, subjects aged 55 and older from the Rotterdam Eye Study II, subjects aged 45 and older from the Rotterdam Eye Study III, and subjects aged 18-86 from the ERF study, resulting in a total sample size of 16,830 individuals for the myopia analyses and 14,981 for the hyperopia analyses. All individuals were of European ancestry. This study involved meta-analysis of aggregate statistics from multiple studies. Approval was obtained by the local ethics committees for all studies, all studies were conducted according to the principles expressed in the Declaration of Helsinki and informed consent was obtained from the study participants at all study sites.

Study design

GWAS analyses of genotype data imputed to HapMap-II were performed for the traits myopia and hyperopia (adjusted for age at examination, sex and years of education) in 9 studies: the Age-Related Eye Disease Study (AREDS), the "Kooperative Gesundheitsforschung in der Region Augsburg" (KORA, "Cooperative Health Research in the Region of Augsburg"), the Framingham Eye Study (FES), the Ogliastra Genetic Park – Talana (OGP-Talana) study, the Multiethnic Study of Atherosclerosis (MESA) and the Rotterdam Eye Studies RSI, RSII, RSIII and the Erasmus Rucphen Family Study (ERF). The results from these analyses were then combined into a discovery meta-analysis GWAS of each trait. Fixed effects meta-analyses were performed with METAL[22] using p values and the effective sample size for each population. METAL calculates a genomic control value[23] for each population and then adjusts each population's results using the corresponding λ

value. The discovery meta-analysis genome-wide significance threshold was taken to be $5x10^{-8}$.

In an attempt to replicate our discovery meta-analysis results and to increase the power of the analyses using our discovery dataset, we obtained association results from 8 other studies, the Blue Mountains Eye Study (BMES), CROATIA-Split, CROATIA-Vis Island, CROATIA-Korcula studies, the Diabetes Control and Complications Trial (DCCT), and the Orkney Complex Disease Study (ORCADES) (Supplemental Methods), just for 30 genomic regions that contained SNPs with association p-values less than $1x10^{-5}$ to either myopia (11 regions) or hyperopia (14 regions) or both (5 regions) in our discovery meta-analysis (the previously well-replicated association region on chromosome 15q14 was excluded). These studies all performed association of SNPs in these regions with myopia and hyperopia (adjusted for age at examination, sex, years of education when available and up to three principal components when there was significant evidence of population stratification in the data). A replication meta-analysis was performed using the same methods as above on association results in the novel genome-wide significant region for the hyperopia trait in these 8 additional datasets. An additional meta-analysis was then performed in these 30 regions combining results from the discovery datasets and these 8 additional studies. All 8 of these additional datasets were part of the Verhoeven et al. study of mean spherical equivalent. This additional analysis and these datasets are described in Supplemental Materials.

Quality control of discovery datasets

AREDS and KORA: Quality control measures are described elsewhere[24] but in brief: Individuals with chromosome abnormalities and sex discrepancies were removed. Cryptic relatedness was estimated by calculating pairwise identical by descent (IBD) coefficients. For each pair with a kinship coefficient of 0.125 or greater, one member of the pair was dropped based on genotyping rate and trait phenotype, preferring to retain the person with higher genotyping rates and more extreme phenotypes. Population stratification was assessed using principal components. Batch effects and patterns of missingness were eliminated by testing each batch against the others using Fisher's Exact test. As AREDS was a multi-center study, we also tested for differences between collection sites. Samples were dropped for poor performance on the array or a genotyping rate of < 98%. SNPs were also removed from a population if its call rate was below 99%, its minor allele frequency was below 0.01, or if its distribution departed significantly from Hardy-Weinberg expectations ($p<1x10^{-4}$) in a single population. We additionally dropped SNPs in both populations where HWE $p < 1x10^{-4}$ in 1 population and HWE p < 1x10-3 in the other. SNPs were also excluded if they showed more than one genotype inconsistency between HapMap control samples and the consensus genotype in the HapMap database or investigator-provided duplicate samples.

Framingham Eye Study: Quality control measures are described elsewhere[24] but in brief: Samples were chosen based on pedigree information and genotyping quality. Samples with a genotypic call rate below 95% were not chosen for analysis. The mean call rate for analyzed samples was 99.2% (SD=0.4%). The final marker list contained 436,494 high-quality SNPs with a minor-allele frequency >= 0.01, a Mendelian error rate below 2% across all pedigrees, a genotype call rate above 95%, and whose distribution was consistent with Hardy-Weinberg expectations ($P>1x10^{-4}$).

MESA: For the MESA dataset, SNPs with MAF less than 0.02 or HWE p value less than 0.001 were

removed from the analysis. Genotyping was performed using the Affymetrix Genome-Wide Human SNP Array 6.0. IMPUTE version 2.1.0 was used to perform imputation for the MESA Caucasian participants (chromosomes 1-22) using HapMap Phase I and II - CEU as the reference panel (release #24 - NCBI Build 36 (dbSNP b126)). SNPs with genotype call rate less than 0.95, MAF less than 0.02, HWE p value less than 0.001, or oevar less than 0.3 were removed from the analysis. Association tests were performed by SNPTEST v2 (Marchini et al, 2007).

OGP-Talana: Quality control of the SNP data was performed using the GenABEL software package in R. Samples with overall SNP call rate < 93%, with minor allele frequency < 0.01, with Hardy-Weinberg P value $>10^{-6}$, showing excess heterozygosity, or being classified as outliers by allelic identity-by-state (IBS) clustering analysis, were excluded.

Rotterdam Eye Studies I,II and III: Subjects with cataracts and history of cataract or refractive surgery were excluded from the study. DNA was extracted from blood leucocytes according to standard procedures. Genotyping of SNPs was performed using the Illumina Infinium II HumanHap550 chip v3.0 array (RS-I); the HumanHap550 Duo Arrays and the Illumina Human610-Quad Arrays (RS-II), and the Illumina Human 610 Quad Arrays (RS-III). Samples with low call rate (<97.5%), with excess autosomal heterozygosity (>0.336), or with sex-mismatch were excluded, as were outliers identified by the identity-by-state clustering analysis (outliers were defined as being >3 s.d. from population mean or having identity-by-state probabilities >97%). GWAS analyses were performed using GRIMP.

Erasmus Rucphen Family Study: Subjects with cataracts and history of cataract or refractive surgery were excluded from the study. DNA was genotyped on one of four different platforms (Illumina 6k, Illumina 318K, Illumina 370K and Affymetrix 250K). Samples with low call rate (<97.5%), with excess autosomal heterozygosity (>0.336), or with sex-mismatch were excluded, as were outliers identified by the identity-by-state clustering analysis (outliers were defined as being >3 s.d. from population mean or having identity-by-state probabilities >97%). GWAS analyses were performed using the ProbABEL package from the ABEL set. A lambda correction was performed to adjust for cryptic relationship.

Genotype imputation of data

To produce a consensus set of genotypes for imputing to the HapMap-II, AREDS and KORA high quality SNPs were filtered to those present on HapMap-II. Imputation to the HapMap-II reference panel (CEU population release 22, NCBI build 36) was performed in MACH[22,25] in 2 stages. Stage one was the model parameter estimation stage which used a random sample of 300 individuals from each population, using the greedy option which only uses the reference haplotypes (supplied here from the HapMap) and 100 Markov Chain iterations. Stage two is the actual imputation stage and uses the model parameters estimated in stage one to speed up the imputation of the genotypes. After imputation, the remaining high quality genotyped SNPs were merged back in with the SNPs from the imputation procedure for the AREDS and KORA data. For the FES data, genotype imputation to the HapMap-II reference panel (CEU population release 22, NCBI build 36) was carried out in a two-step process using the Markov Chain Haplotyping (MACH version 1.0.16.a) software. First, crossover and error-rate maps were built using 400 unrelated

individuals (200 male and 200 female) sampled from FHS subjects. Second, genotype imputations of approximately 2.5 million autosomal HapMap-II SNPs were carried out on the entire FHS dataset using parameters estimated from step 1. For MESA, IMPUTE version 2.1.0 was used to perform imputation for the Caucasian participants (chromosomes 1-22) using HapMap Phase I and II - CEU as the reference panel (release #24 - NCBI Build 36 (dbSNP b126)). For OGP-Talana, using the phase II CEU HapMap individuals (release 22, NCBI build 36) as reference panel for imputation, genotypes were imputed for nearly 2.5 million SNPs using MACH. SNPs imputed with Rsq <0.3 were excluded. For RSI,II and III and ERF, a set of genotyped input SNPs with call rate >98%, with minor allele frequency >0.01, and with Hardy-Weinberg P value $>10^{-6}$ was used for imputation. We used the Markov Chain Haplotyping (MACH) package version 1.0.15 software (Rotterdam, The Netherlands; imputed to plus strand of NCBI build 36, HapMap release #22) for the analyses. For each imputed SNP, a reliability of imputation was estimated as the ratio of the empirically observed dosage variance to the expected binomial dosage variance (O/E ratio).

Data analysis

Genetic association was estimated by fitting a logistic regression model separately to the traits myopia and hyperopia. To create the dichotomous traits, we calculated mean spherical equivalent (MSE) as the average of spherical equivalent (SE) of refraction between the two eyes, or the single SE value for persons with only a single SE measurement. For myopia, cases were defined as MSE < -1D, controls > 0D and individuals between 0D and -1D coded as unknown. For hyperopia, cases were defined as MSE >+1D, controls < 0D and individuals between 0D and +1D coded as unknown. A general additive genetic model was used to code the SNP effect (i.e. SNPs were coded according to the number of minor alleles [0,1,2] for each person); covariates included age; sex; and years of education. For AREDS, KORA and FES, this was accomplished using the PLINK (version 1.07) statistical software (http://pngu.mgh.harvard.edu/~purcell/plink)[26]. For AREDS analyses, the first three principal components (eigenvectors) of the EIGENSTRAT analysis were also included along with the covariates listed above. For MESA, these association tests were performed by SNPTEST v2.52. For OGP-Talana, all regression models were run using the ProbABEL package from the ABEL set of programs which adjusts jointly for cryptic relationship and population stratification. For RSI, II and III and ERF, we used genomic control[23] to obtain optimal and unbiased results and applied the inverse variance method of each effect size estimated for both autosomal SNPs that were genotyped and imputed in both cohorts.

Association analyses were performed for both traits and a genome-wide meta-analysis was performed on the 9 populations and 8 replication data sets (Blue Mountains Eye Study, Croatia Vis Island Study, Croatia Korcula Study, Diabetes Control and Complications Trial, Orkney Complex Disease Study, UK Twins Study, 1958 British Birth Cohort, Wisconsin Epidemiologic Study of Diabetic Retinopathy). Details of the genome-wide analyses of the individual discovery datasets and the replication analyses are shown in the supplemental methods and results including QQ-plots and Manhattan plots for each of the discovery cohorts in Supplementary Figures 1-9. Supplementary Figure 10 is a flowchart showing the workflow of the entire study.

SNP selection for replication

Thirty genomic regions that contained SNPs with association p-values less than $1x10^{-5}$ to either myopia (11) or hyperopia (14) or both (5) in our discovery meta-analysis (excluding the 15q14 region) were chosen for replication or further study in the 8 additional datasets. We analyzed all SNPs within a 500 kb window centered on the most significant SNP in each region from the discovery meta-analysis.

For the comparison of our discovery meta-analysis results with the myopia age at onset loci from the Kiefer et al[18] study, a list of strongly associated variants that were genome-wide significant ($p \leq 5x10^{-8}$) or suggestive ($p < 1x10^{-6}$) in Kiefer et al[18] was selected. We analyzed all SNPs within a 500 kb window centered on these replication SNPs in our data.

Calculation of effective number of tests and replication significance thresholds

It has become increasingly clear that only attempting to replicate the exact SNPs found to be genome-wide significant in a discovery GWAS can produce a failure to replicate due to underlying differences in linkage disequilibrium (LD) and allele frequencies[27,28], even in populations self-identified as having the same ethnicity. Ioannidis et al.[29] have shown that restricting replication efforts to only a few of the most significant SNPs from an associated region leads to less robust information for those loci. The resulting failure to replicate may be because those selected SNP(s) are not necessarily more informative or closer to the causal variant than other SNPs in the region. Several approaches to this problem have been proposed, including incorporating linkage information[30], pathway-based association[31] and other methods which use multiple SNPs in the analysis[32-39]. A linkage disequilibrium (LD) based binning strategy, proposed by Christoferou[39] may prove to be the most useful. However, the issues of handling SNPs which map to more than one gene due to overlapping reading frames and the correlations between genes and derivative gene scores still need to be resolved. Until that problem has a solution, it may be more powerful to study a dense panel of SNPs from each associated region, and utilize imputation to the latest version of 1000 Genomes data to provide additional genotypes to harmonize available SNPs across studies even when genotyped on different platforms. Here we selected all SNPs that were within a specified window of the original SNP and used the method of Ramos et al[40] to model the LD structure in one of the replication populations to calculate the effective number of independent tests being performed across all of our replication regions. Traditional methods of correcting for multiple comparisons, such as the widely used Bonferroni correction considering all SNPs tested, are notoriously conservative because they do not take intermarker correlation fully into account but treat all the tests as independent. By using the effective number of independent tests in a Bonferroni correction, Type I error is still controlled and power is improved. Various approaches to calculating the effective number of independent tests when using such a regional replication strategy have been proposed since many of the SNPs in such a region are in LD with each other and do not represent independent tests[41-45], although many of these approaches are still overly conservative. The Ramos et al.[40] approach properly accounts for SNP interdependence, allows computation of the effective number of independent tests for very large numbers of highly correlated SNPs and is less computationally intensive than permutation-based methods. We used the method of Ramos et al[40] to calculate the number of effective tests (N_{eff}) in all the replication regions and divided α

by this effective number of tests to calculate the significance threshold separately in the AREDS, KORA and Framingham datasets. The Ramos method calculates (N_{eff}) by first estimating the KxK covariance matrix for the K SNPs in the replication regions using the genotype data. Then the covariance matrix is spectrally decomposed to calculate the eigenvalues. The effective number of tests is then estimated using the relationship

$$N_{eff} = \left(\sum_{k=1}^{K} \lambda_k \right)^2 / \left(\sum_{k=1}^{K} \lambda_k^2 \right)$$

in which λ_k is the kth eigenvalue of the K×K covariance matrix for the K SNPs[46]. The Bonferroni-corrected significance threshold is then calculated as α / N_{eff}

The markers in each region are very densely spaced, with high levels of LD between markers in each block. The calculations from the AREDS data gave the largest effective number of tests and thus the most conservative Bonferroni-corrected significance threshold; thus this was chosen as our significance threshold for our replication studies. However, the Bonferroni-corrected thresholds derived by applying this method to the KORA and Framingham data were only slightly less conservative than the threshold derived from the AREDS data.

RESULTS

After all quality control measures and appropriate association analyses, genome-wide association results from Caucasian participants in the AREDS, KORA, FES, OGP-Talana, MESA, RSI, RSII, RSIII and ERF studies were combined in a genome-wide discovery meta-analysis totaling 16,830 individuals for myopia and 14,981 individuals for hyperopia. Table 1 describes the characteristics of the populations after classifying participants into myopia, hyperopia, control or unknown categories.

Testing for population stratification using EIGENSOFT and principal components analysis found no evidence of population stratification in KORA, but some evidence of substructure was detected in the AREDS, FES and MESA studies. These were adjusted for in the genome-wide association analyses by including the first three principal components from the PCA as covariates in our regression models. The OGP-Talana data were also adjusted for cryptic relatedness using the ProbABEL R package. For ERF and RS I-III, the population was assumed to be homogeneous and outliers excluded. Genomic control[23] values (λ) calculated by METAL[47] for each population prior to meta-analysis for each trait are given in Table 1. These values were used by METAL to adjust each population's results before including in the fixed effects meta-analysis. The QQ plots of the meta-analysis p values (Figure 1A and Figure 2A) showed some deviation from the null. However, the genomic control method[23] was used to further control for population stratification and inter-population differences in the final meta-analysis. The variance inflation factors calculated by METAL[47] for the final meta-analysis across the nine cohorts for myopia and hyperopia were 1.038 and 1.046 respectively. Lambda values ranging from approximately 0.95 to 1.1 are considered desirable.

Table 1. Baseline characteristics of the nine populations

	AREDS	KORA	FES	MESA	OGP-Talana	RS-I	RS-II	RS-III	ERF	**Total**
N	1877	1869	1389	1462	683	5238	2009	1970	2028	18525
Mean Age (SD)	68.0 (4.7)	55.6 (11.8)	55.6 (8.9)	61.9 (9.4)	42.2 (19.1)	68.5 (8.6)	64.2 (7.4)	60.8 (5.5)	48.5 (14.3)	
N Myopia[1] Cases	346	550	348	486	71	763	395	594	370	3923
Myopia Cases MSE[2]	-2.81	-2.72	-3.08	-3.20	-4.41	-3.21	-3.08	-3.22	-3.03	
N Myopia Controls	1333	840	773	731	428	3964	1374	1056	1197	11696
Myopia Controls MSE[2]	1.59	1.38	1.60	1.70	1.38	1.88	1.72	1.39	1.24	
N Myopia Unknown	198	479	268	245	184	601	240	320	461	
N Hyperopia[3] Cases	854	424	426	506	64	2779	919	556	540	7068
Hyperopia Cases MSE[2]	2.56	2.30	2.31	2.21	2.42	2.48	2.33	2.23	2.29	
N Hyperopia Controls	600	1010	654	714	153	1350	627	907	829	6844
Hyperopia Controls MSE[2]	-1.76	-1.79	-1.92	-2.32	-2.56	-2.00	-2.09	-2.21	-1.52	
N Hyperopia Unknown	423	435	309	242	466	1109	463	507	659	
Sex (% Male) (Myopia/Hyperopia)	41/40	50/49	42/41	54/43	41/40	48/39	49/44	46/43	53/62	
Myopia	1.001	0.997	1.004	1.024	1.085	1.020	1.018	1.015	1.323	1.038
Hyperopia	1.020	1.023	0.997	1.017	1.156	1.041	1.024	1.010	1.254	1.046

1. For myopia, cases were defined as MSE < -1D, controls > 0D and individuals between 0D and -1D coded as unknown.
2. Average MSE of all cases or controls used in the analyses.
3. For hyperopia, cases were defined as MSE >+1D, controls < 0D and individuals between 0D and +1D coded as unknown.

Results of the genome-wide meta-analyses are shown in Figure 1B and Figure 2B and results for each sample separately are given in Supplemental Figure 1 (AREDS), Supplemental Figure 2 (KORA), Supplemental Figure 3 (FES), Supplemental Figure 4 (MESA), Supplemental Figure 5 (OGPT), Supplemental Figure 6 (RS-I), Supplemental Figure 7 (RS-II), Supplemental Figure 8 (RS-III), Supplemental Figure 9 (ERF). Eight additional studies (1958 British Birth Cohort, BMES, CROATIA-Vis, CROATIA-Korcula, DCCT, ORCADES, TwinsUK and WESDR) were used for replication and baseline characteristics of these studies can be found in Supplemental Table S3. Results of further meta-analyses of genomic regions that exhibited suggestive evidence of association with myopia or hyperopia using regional results from the 8 additional studies listed above are given in Supplemental Tables S6 and S7. Meta-analyses combining the replication region association results from the 9 discovery datasets and the 8 replication datasets did not result in genome-wide significant results, except for the 8q12 locus (results not shown) that was already genome-wide significant in the discovery dataset.

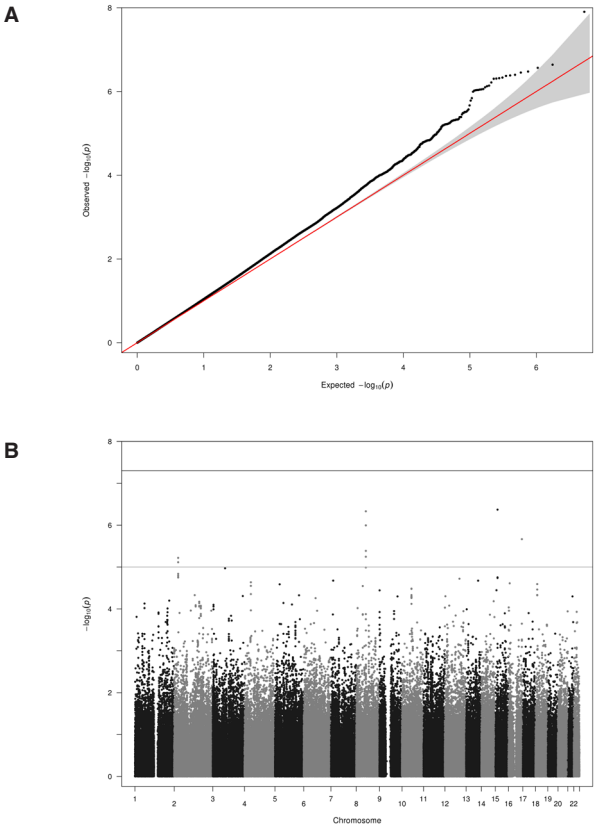

Figure 1. QQ and Manhattan plots for the myopia analysis of all cohorts. A. QQ plot for association between all SNPs analyzed and myopia in the meta-analysis. Each dot represents an observed statistic (defined as -log10 P) versus the corresponding expected statistic. The red line corresponds to the null distribution. B. Manhattan plot for association between all SNPs analyzed and myopia in the meta-analysis. Each dot represents an observed statistic (defined as -log10 P). The darker gray line corresponds to the genome-wide significance threshold and the lighter gray line represents the suggestive threshold.

To determine if our discovery meta-analyses showed evidence of association in any of 35 loci (Supplemental Table S1) reported to exhibit genome-wide significant or suggestive ($p < 1x10^{-6}$) association with myopia age at onset by Kiefer et al[18], a total of 33,591 SNPs overlapping all associated loci were selected (Supplemental Table S2). These included the most significant discovery SNP plus all available genotyped and imputed SNPs within 500kb of the most significant discovery SNP (Supplemental Table S2). Accounting for all the LD in each region reduced the effective number of tests, N_{eff}, to 475.71. The replication significance threshold, calculated while taking into account this LD structure in replication regions[40], was $\frac{a}{N_{eff}} = \frac{0.05}{475.71} = 0.0001$.

Myopia

Results of the discovery meta-analysis (Figure 1, Supplemental Table S4) shows one genome-wide significant marker corresponding to a previously identified myopia age at onset[18] and refractive error[19] locus on 8q12 (rs10113215, $p=1.25x10^{-8}$). We also observed association to the well-replicated locus on 15q14 (near *GJD2*) that was close to genome-wide significant (rs1370156, $p=2.29x10^{-7}$). No attempt was made to replicate the chromosome 15q14 region since it has been well replicated. SNPs in the 8q12 replication region did not reach the replication threshold (for rs10113215, replication p=0.02; top replication p-value in the region was p=0.0022 for rs6995115). For the discovery meta-analysis suggestive regions, one of the selected SNPs achieved the replication threshold for myopia (rs4326350 on 8p23, $p=6.1x10^{-5}$). However, it should be remembered that this region did not exhibit genome-wide significant association in the discovery meta-analysis (replication p-values in Supplementary Table S6).

In addition to the 8q12 locus, 10 other myopia age at onset regions from the Kiefer et al study[18] showed significant evidence of replication in our discovery meta-analysis (Table 2). Eight of these loci have also been reported as associated with MSE by Verhoeven et al [19]. However, two of the regions we replicated were not reported significantly associated with MSE by Verhoeven et al[19]. On chromosome 3p26, rs2587916 reached the replication threshold in our discovery meta-analysis ($p=2.79x10^{-5}$). This SNP is 256bp away from the SNP reported in this region by Kiefer et al[18], rs1843303 (which had $p=6.32x10^{-4}$ in our data, Table 2). These two SNPs exhibit strong linkage disequilibrium with an R^2 of 0.963 and a D′ of 1 in our data. The most significant SNP at the second locus on chromosome 6 is the same SNP as reported by Kiefer et al[18], rs7744813 ($p=6.07x10^{-6}$, Table 2).

Due to the high genomic control values for OGP-Talana and ERF (Table 1), we examined QQ plots of only the common SNPs (MAF > 0.2) to see if this made an improvement, since all the associated SNPs reported here have high MAFs. In OGP-Talana this improved the QQ plots (Supplementary Figure 9) but it made no difference for ERF. Therefore, we dropped ERF from the analysis and re-examined the results (Figure 3). For most loci this made minimal difference to the p values. However, for 3 loci there was a considerable difference. The genome-wide significant result for myopia on chromosome 8 was no longer genome-wide significant ($p=8.8x10^{-7}$), although it still remained well below our replication significance threshold. The loci on 2q37 and 3p26 were no longer below our replication threshold.

Table 2. Results of the replication of regions significantly associated with myopia age at onset by Kiefer et al[18] showing meta-analysis association results for each chosen SNP with myopia in our data

Replication SNP[1]	Chromo-some	Position	Replication P value[2]	Best SNP[3,6]	Offset[4,6]	P value[5,6]	Nearest Gene(s)[7]	Reported by Verhoeven et al
rs6702767	1	200844547	1.12E-01	rs4471299	391129	1.92E-04		No
rs11681122	2	146786063	NA	rs10928276	661	4.61E-04		No
rs17428076	2	172851936	7.13E-02	rs3821093	157350	7.50E-03		No
rs1898585	2	178660450	NA	rs1405645	192929	1.47E-03		No
rs1550094	**2**	**233385396**	**NA**	**rs1656404**	**5456**	**3.72E-05**	***PRSS56***	Yes
rs1843303	**3**	**4185124**	**6.32E-04**	**rs2587916**	**256**	**2.79E-05**	***SUMF1/ SETMAR***	No
rs7624084	3	141093285	2.93E-02	rs1007118	247701	3.53E-03		No
rs1031004	4	80516849	NA	rs1440853	10203	4.09E-04		No
rs5022942	**4**	**81959966**	**NA**	**rs1353387**	**12783**	**6.16E-05**	***BMP3***	Yes
rs7744813	**6**	**73643289**	**6.07E-06**				***KCNQ5***	No
rs12193446	**6**	**129820038**	**8.74E-06**				***LAMA2***	Yes
rs9365619	6	164251746	5.26E-01	rs6900149	211224	2.34E-02		No
rs2137277	**8**	**40734662**	**2.84E-05**	**rs4736884**	**5031**	**1.78E-05**	***ZMAT4***	Yes
chr8:60178580	**8**	**60178580**	NA	**rs10113215**	**46386**	**1.25E-08**	***TOX***	Yes
rs10963578	9	18338649	NA	rs10115405	17893	8.99E-04		No
rs11145746	9	71834380	1.12E-02	rs3002374	35408	2.88E-04		No
rs4245599	**10**	**60365755**	**5.75E-05**	**rs12264028**	**87616**	**2.57E-05**	***BICC1***	Yes
rs6480859	10	79081948	5.36E-02	rs16933964	457642	1.00E-03		No
rs745480	10	85986554	6.88E-03	rs4244950	34147	2.12E-04		No
rs4367880	10	114795256	NA	rs7071843	316234	1.11E-03		No
rs11602008	11	40149305	NA	rs7924805	61948	1.02E-03		No
chr11:65348347	11	65348347	NA	rs610037	198510	5.94E-03		No
rs10736767	11	84637065	6.61E-02	rs1940124	18791	6.49E-04		No
rs6487748	12	9435768	NA	rs12822596	125774	1.83E-03		No
rs3138142	12	56115585	6.68E-02	rs2291615	219566	3.18E-03		No
rs4291789	**13**	**100672921**	**NA**	**rs8000506**	**3929**	**2.98E-05**	***ZIC2/ ZIC5***	Yes
rs61988414	14	42313443	NA	rs12878452	2013	1.61E-03		No
chr14:54413001	14	54413001	NA	rs12147340	493078	1.43E-03		No
rs524952	**15**	**35005886**	**8.74E-05**	**rs1370156**	**21004**	**2.29E-07**	***GJD2***	Yes
rs4778882	15	79382019	NA	rs925114	323501	6.84E-04		No
rs17648524	**16**	**7459683**	**3.03E-06**	**rs4581716**	**1549**	**1.65E-06**	***RBFOX1***	Yes
rs2908972	17	11407259	4.10E-03	rs4792105	295899	1.79E-03		No
rs10512441	17	31239645	2.47E-03	rs17780981	120609	5.52E-04		No
rs9902755	17	47220726	1.51E-01	rs7222737	31323	2.16E-03		No
chr17:79585492	17	79585492	NA	rs11651296	232337	8.53E-03		No

1. SNPs which are either genome-wide significant or meet our replication threshold are highlighted in bold text. Allele frequencies for these SNPs in each of our discovery populations can be found in Supplemental Table S8.
2. For each SNP reported by Kiefer et al, Replication P value is the P value of that SNP in our analysis. If that SNP was not genotyped or imputed in our data, it is indicated with NA.
3. For regions where the most significant SNP in our analysis is not the original reported SNP, that SNP is reported as Best SNP.
4. Offset is the absolute distance in base pairs to the original SNP and the P value associated with Best SNP.
5. Z scores and direction of effect for all SNPs are in Supplemental Table S2.
6. This column left blank where the original SNP is the most significant SNP in the region.
7. Nearest Gene(s) indicates the closest gene by physical position for these SNPs.

Hyperopia

Meta-analysis results showed two genome-wide significant associations with hyperopia (Figure 2, Supplemental Table 5). These regions overlapped with loci on 15q14 (rs11073060, p=9.11x10^{-11}) and 8q12 (rs10089517,p=1.82x10^{-11}) previously reported for MSE in Verhoeven et al[19] and for myopia age at onset in Kiefer et al[18]. No attempt was made to replicate the 15q14 locus since it has been well replicated for MSE. None of the SNPs selected to attempt replication of the discovery meta-analysis genome-wide significant association with hyperopia on chromosome 8q12 achieved the replication threshold (rs10089517, p=0.08; top replication p-value in the region was 0.014 at rs11778476) (Supplementary Table S7). In addition, for the discovery meta-analysis suggestive regions, one SNP achieved the replication threshold for hyperopia (rs12660628 on 6q21, p=7.7x10^{-5}). However, it should be remembered that this region did not exhibit genome-wide significant association in the discovery meta-analysis (replication p-values in Supplementary Table S7).

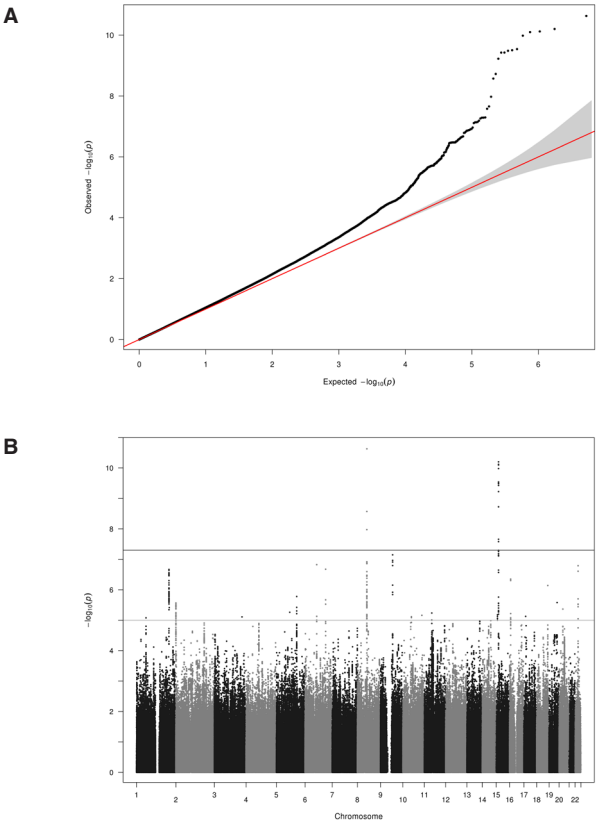

Figure 2. QQ and Manhattan plots for the hyperopia analysis of all cohorts. A. QQ plot for association between all SNPs analyzed and hyperopia in the meta-analysis. Each dot represents an observed statistic (defined as -log10 P) versus the corresponding expected statistic. The red line corresponds to the null distribution. B. Manhattan plot for association between all SNPs analyzed and hyperopia in the meta-analysis. Each dot represents an observed statistic (defined as -log10 P). The darker gray line corresponds to the genome-wide significance threshold and the lighter gray line represents the suggestive threshold.

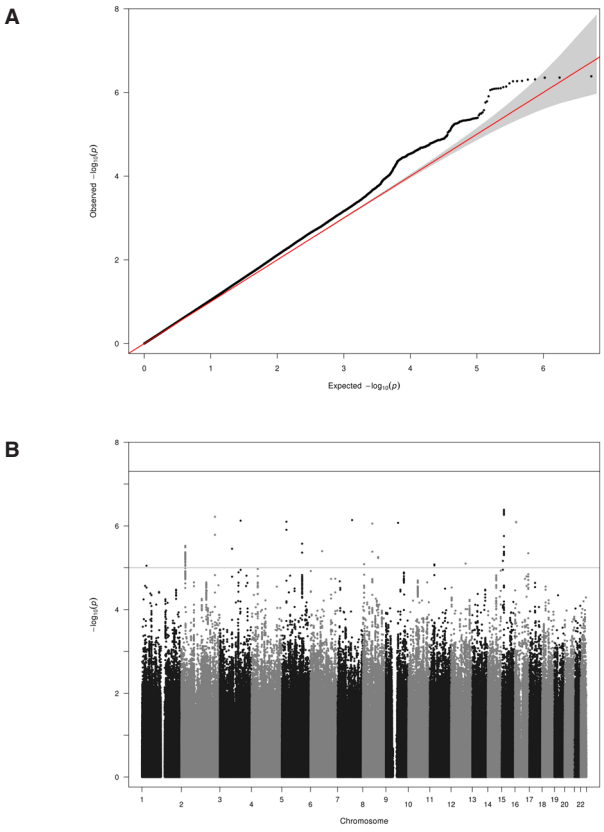

Figure 3. QQ and Manhattan plots for the myopia analysis excluding the ERF cohort. A. QQ plot for association between all SNPs analyzed and myopia in the meta-analysis excluding the ERF cohort. Each dot represents an observed statistic (defined as -log10 P) versus the corresponding expected statistic. The red line corresponds to the null distribution. B. Manhattan plot for association between all SNPs analyzed and myopia in the meta-analysis excluding the ERF cohort. Each dot represents an observed statistic (defined as -log10 P). The darker gray line corresponds to the genome-wide significance threshold and the lighter gray line represents the suggestive threshold.

In addition to the 15q14 and 8q12 loci, 10 other regions (Table 3) that were genome-wide significant in the Kiefer et al[18] analysis of myopia age at onset exhibited p values for association with hyperopia that met our "replication" threshold for these regions. Given this is a different but related trait, this finding is interesting. Five of these regions have been replicated using myopia as the trait in our data here (three of which were also found to be significantly associated with MSE by Verhoeven et al[19] also found that 1 more of these 10 regions (Table 3) showed significant association with MSE. Of the remaining 4 regions from Table 3 the most significant of these 4 SNPs was rs1371993 ($p=1.13x10^{-5}$), a SNP on chromosome 4, 35Kb from the SNP reported by Kiefer et al[18] for myopia age at onset (rs1031004, not available in our data).

Due to the high genomic control values for OGP-Talana and ERF (Table 1), we examined QQ

Table 3. Results of the hyperopia analyses in the regions that were significantly associated with myopia age at onset by Kiefer et al[18] showing meta-analysis association results for each chosen SNP

Replication SNP[1]	Chromosome	Position	Replication P value[2]	Best SNP[3,6]	Offset[4,6]	P value[5,6]	Nearest Gene(s)[7]	Reported by Verhoeven et al
rs6702767	1	200844547	1.60E-01	rs6703834	264384	4.58E-03		No
rs11681122	2	146786063	NA	rs17412774	12116	1.50E-04		No
rs17428076	2	172851936	6.43E-03	rs3821093	157350	2.44E-04		No
rs1898585	**2**	**178660450**	**NA**	**rs6718702**	**84399**	**1.47E-05**	***PDE11A***	No
rs1550094	**2**	**233385396**	**NA**	**rs1881494**	**12631**	**4.63E-05**	***PRSS56***	Yes
rs1843303	**3**	**4185124**	**1.98E-05**	**rs795294**	**826**	**1.18E-05**	***SUMF1/ SETMAR***	No
rs7624084	3	141093285	NA	rs9821337	2901	1.88E-04		No
rs1031004	**4**	**80516849**	**NA**	**rs1371993**	**35034**	**1.13E-05**	***GK2*** **(OMIM #137028)**	No
rs5022942	4	81959966	NA	rs2201544	30290	4.94E-03		Yes
rs7744813	**6**	**73643289**	**7.00E-08**				***KCNQ5***	No
rs12193446	**6**	**129820038**	**1.84E-07**				***LAMA2***	Yes
rs9365619	6	164251746	2.67E-01	rs2759387	412079	9.50E-03		No
rs2137277	8	40734662	2.72E-02	rs6474290	94596	2.42E-03		Yes
chr8:60178580	**8**	**60178580**	**NA**	**rs10089517**	**141**	**1.82E-11**	***TOX***	Yes
rs10963578	9	18338649	NA	rs10115405	17893	2.54E-04		No
rs11145746	9	71834380	8.33E-03	rs10481782	22378	2.71E-04		No
rs4245599	10	60365755	1.16E-03	rs1866168	4194	8.11E-04		Yes
rs6480859	10	79081948	1.45E-02	rs16933964	457642	4.35E-04		No
rs745480	10	85986554	3.26E-01	rs17103281	25190	1.06E-04		No
rs4367880	10	114795256	NA	rs7914029	215000	3.40E-04		No
rs11602008	**11**	**40149305**	**NA**	**rs10837366**	**75045**	**7.61E-05**	***LRRC4C*** **(OMIM #608817)**	No
chr11:65348347	11	65348347	NA	rs11820062	81589	7.56E-03		No
rs10736767	11	84637065	1.99E-01	rs10898278	303825	3.05E-03		No
rs6487748	12	9435768	NA	rs7305636	157088	9.29E-04		No
rs3138142	12	56115585	4.32E-02	rs12828230	230568	5.87E-04		No
rs4291789	**13**	**100672921**	**NA**	**rs1347190**	**24823**	**6.65E-06**	***ZIC2/ZIC5***	Yes
rs61988414	14	42313443	NA	rs10149831	125528	1.35E-03		No
chr14:54413001	14	54413001	NA	rs17127526	444960	1.26E-03		No
rs524952	**15**	**35005886**	**3.07E-08**	**rs11073060**	**16036**	**9.11E-11**	***GJD2***	Yes
rs4778882	15	79382019	NA	rs1443658	4348	2.88E-03		No
rs17648524	**16**	**7459683**	**4.86E-07**				***RBFOX1***	Yes
rs2908972	**17**	**11407259**	**1.39E-04**	**rs12602611**	**166838**	**1.26E-05**	***SHISA6***	No
rs10512441	17	31239645	4.78E-03	rs17183113	210521	2.40E-03		No
rs9902755	17	47220726	2.81E-01	rs8064938	439898	1.73E-03		No
chr17:79585492	17	79585492	NA	rs6565596	60374	1.13E-02		No

1. SNPs which are either genome-wide significant or meet our replication threshold are highlighted in bold text. Allele frequencies for these SNPs in each of our discovery populations can be found in Supplemental Table S8.
2. For each SNP reported by Kiefer et al, Replication P value is the P value of that SNP in our analysis. If that SNP was not genotyped or imputed in our data, it is indicated with NA.
3. For regions where the most significant SNP in our analysis is not the original reported SNP, that SNP is reported as Best SNP.
4. Offset is the absolute distance in base pairs to the original SNP and the P value associated with Best SNP.
5. Z scores and direction of effect for all SNPs are in Supplemental Table S2.
6. This column left blank where the original SNP is the most significant SNP in the region.
7. Nearest Gene(s) indicates the closest gene by physical position for these SNPs.

plots of only the common SNPs (MAF > 0.2) to see if this made an improvement, since all the SNPs reported here have high MAFs. In OGP-Talana this improved the QQ plots (Supplementary Figure 9) but it made no difference for ERF. Therefore, we dropped ERF from the analysis and re-examined the results (Figure 4). For all loci this made minimal difference to the p values and did not change the conclusions.

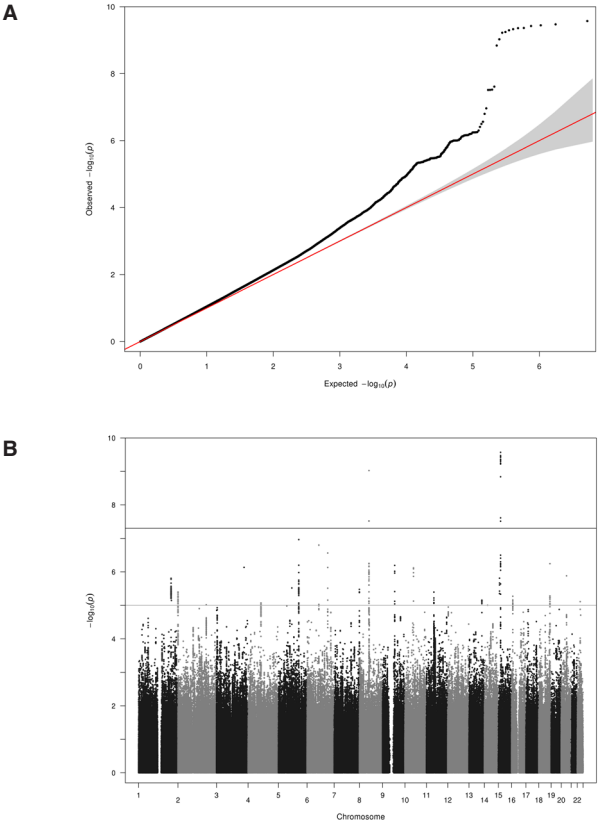

Figure 4. QQ and Manhattan plots for the hyperopia analysis excluding the ERF cohort A. QQ plot for association between all SNPs analyzed and hyperopia in the meta-analysis excluding the ERF cohort. Each dot represents an observed statistic (defined as -log10 P) versus the corresponding expected statistic. The red line corresponds to the null distribution. B. Manhattan plot for association between all SNPs analyzed and hyperopia in the meta-analysis excluding the ERF cohort. Each dot represents an observed statistic (defined as -log10 P). The darker gray line corresponds to the genome-wide significance threshold and the lighter gray line represents the suggestive threshold.

DISCUSSION

We conducted a meta-analysis of 9 myopia and hyperopia genome-wide association studies. We detected the known loci on chromosomes 8q12 and 15q14. The locus on chromosome 8q12 has been reported associated with mean spherical equivalent in an analysis which included many

of the cohorts in this study[19], and myopia age at onset in an independent study[18]. The locus on chromosome 15q14 was discovered in some of the cohorts included in this analysis[48] and has been well replicated in studies of both MSE[19] and myopia age at onset[18]. These findings were therefore expected. However, the signal for 15q14 is only genome-wide significant in the hyperopia analysis here. In addition, although the 8q12 locus was genome-wide significant in the myopia analysis, it was more significant in the hyperopia analysis. Nonetheless, the direction of effect of these SNPs is exactly opposite in the myopia and hyperopia analyses – suggesting that the causal mechanisms being tagged by these SNPs are operating across the spectrum of refractive error.

We also examined the results of our discovery meta-analyses of myopia (which were adjusted for age at examination and years of education) to attempt targeted "replication" of 35 GWAS-identified loci that have previously been reported by Kiefer et al to be associated with age at onset of myopia. Since age at onset was not available in all our study samples, it was not possible to perform an exact replication of the Kiefer et al[18] trait on which they performed survival analysis of myopia age at onset. Our analyses, where we included age at exam and years of education, is the closest phenotype we had available. We also examined evidence for association with hyperopia in these same regions of the genome, since myopia and hyperopia represent opposite ends of the distribution of refractive error. It is reasonable that loci that affect the variability of MSE as a whole may therefore affect risk of both myopia and hyperopia.

Our analysis provides evidence for replication of a number of loci identified by Kiefer et al[18]. Those which were replicated using the myopia trait (Table 2) represent the closest phenotype available from all of our samples to the one used in their analysis. In particular, this study presents the first report of replication of 11 regions associated with myopia. Of note, nine of these regions also showed genome-wide significant evidence of association to MSE by Verhoeven et al[19]: chromosome 2 near *PRSS56* (OMIM #609995), chromosome 4 near *BMP3* (OMIM #112263), chromosome 6 near LAMA2 (OMIM #156225), chromosome 8 near *ZMAT4* (40734662 bp), chromosome 8 near *TOX* (OMIM #606863, 60178580 bp), chromosome 10 near *BICC1* (OMIM #612717), chromosome 13 near *ZIC2* (OMIM #603073)/*ZIC5*, chromosome 15 near *GJD2* (OMIM #607058) and chromosome 16 near *RBFOX1* (OMIM #605104). The candidate genes in these 9 regions have been discussed by both Kiefer et al[18] and Verhoeven et al[19]. The two remaining Kiefer et al. loci that were not reported as significantly associated with MSE in Verhoeven et al[19] were on 3p26.1 and 6q13. The SNP reported by Kiefer et al[18] in the 3p26.1 region did not meet our replication threshold but another SNP, only 256bp away and in strong linkage disequilibrium with this SNP, did meet our threshold. Kiefer et al[18] proposed the nearby gene *SETMAR* (OMIM #609834), a histone methylation and DNA repair gene as a candidate to explain their observed association with myopia. However, both the SNP detected in our study and the SNP reported by Kiefer et al[18] are intronic to one transcript of *SUMF1* (OMIM #607939), which codes for an enzyme that catalyzes the hydrolysis of sulfate esters. Mutations in this gene are known to cause the lysosomal storage disorder multiple sulfatase deficiency. This multisystem syndrome has been reported to have ocular phenotypes, in the form of retinal degeneration and nystagmus[49]. However, this signal on 3p26.1 was no longer a significant replication when the ERF study results

were removed from the analysis. While the QQ plot of the ERF study results shows some deviation from expected, it does not appear to exhibit overall inflation of the false positive rate for this sample. Thus the replication of this 3p26 locus using all 9 studies may be valid but additional evidence from a larger study will be useful in determining the importance of this locus to risk of myopia. In the 6q13 region, our study replicated the exact same SNP that was reported to have the strongest association with myopia age at onset in the Kiefer et al[18] study and this result did not change with the removal of the ERF study results from our meta-analysis. This associated SNP is in an intron of the *KCNQ5* gene (potassium voltage-gated channel, KQT-like subfamily, member 5, OMIM #607357), which is a member of the *KCNQ* potassium channel gene family. *KCNQ5* has been shown to be differentially expressed in subregions of the brain and in skeletal muscle[50]. Voltage-dependent potassium channels are important regulators of the resting membrane potential and affect the excitability of electrically active cells (OMIM #607357). *KCNQ5* is also expressed in the retinal pigment epithelium (RPE) and neural retina. These potassium channels are believed to affect ion flow across the RPE[51] and the function of cone and rod photoreceptors[51,52].

Other regions that were found to be significantly associated with myopia by Kiefer et al[18] showed some evidence of association with hyperopia but not with myopia in our data. The significance levels of these associations reached our "replication" threshold. This intriguing result suggests that these loci may not be myopia specific. However, much larger sample sizes will be required to further investigate this issue.

One of the Kiefer et al[18] loci that did not replicate in the analysis of myopia and was not previously reported as significantly associated with MSE was a locus on 2q31.2. This locus showed evidence of association with hyperopia in our data that reached our "replication" threshold. Kiefer et al suggested that this association might be due to variants in the phosphodiesterase 11A gene (*PDE11A,* OMIM #604961), which as a known cell signaling molecule is a good candidate gene for development of refractive errors, given the importance of neural signaling in the control of eye growth. However, the signal in our hyperopia analysis stretches across 3 genes: *PDE11A*; tetratricopeptide repeat domain 30A (*TTC30A*) protein; and alkylglycerone phosphate synthase (*AGPS*, OMIM #603051). Mutations in AGPS are associated with rhizomelic chondrodysplasia punctata, type 3, a multisystem developmental disorder in which patients frequently develop cataracts[53].

For the locus on chromosome 4 that showed some evidence of association with hyperopia in our data, Kiefer et al[18] suggested that *ANTXR2* (OMIM #106490), a gene involved in extracellular matrix adhesion was the best candidate, but other good candidates exist in this region such as *BMP2* inducible kinase (*BMP2K*) and annexin A3 (*ANXA3*, OMIM #106490) a gene involved in regulation of cell growth and signal transduction pathways. Two other bone morphogenic proteins whose genes are located elsewhere in the genome have been identified as candidate genes by Kiefer et al[18] and Verhoeven et al[19] and have also been observed in animal models of myopia[54,55]. The role of this group of genes in growth regulation is well known[56].

Given that hyperopia and myopia are the extreme ends of the refractive error distribution, it is tempting to assume that the same risk factors must affect the risk of developing both traits equally.

However, it is not yet clear whether those environmental and genetic factors which increase the risk of developing myopia necessarily affect the risk of hyperopia. The results presented here provide some tantalizing evidence that some genetic factors may be important in both traits whereas others may be more important in driving myopization than hyperopization or *vice versa*. It has now been shown that 9 regions (2q37, 4q21, 6q22, 8p11, 8q12, 10q21, 13q32, 15q14, 16p13) show association to age at onset of myopia[18], myopia adjusted for age at exam, sex and years of education (results presented here) and mean spherical equivalent[19]. However, we observed replication-level association with myopia for an additional 2 loci (6q13 and 8p11) which were not genome-wide significant for mean spherical equivalent[19] but were genome-wide significant for myopia age at onset[18]. An additional four regions that were genome-wide significant in the Kiefer et al analysis of age at onset of myopia[18] have only been "replicated" in our hyperopia analyses. These results indicate that the genetic underpinnings of refractive errors are quite complex and that analyses of both the qualitative and quantitative phenotypes may add to our understanding of refractive error causation. The study participants whose data were analyzed here were not selected for extreme or "high" myopia (typically defined as SE < -6D) and there were very few individuals with high myopia in any of these datasets. Future studies to examine whether any of the loci that show association to myopia, hyperopia and mean spherical equivalent in the population-based studies also show evidence of association to high myopia would be interesting and should be pursued.

Some of the other loci that showed significant association with myopia in the Kiefer et al[18] study did not replicate in our current study. Dichotomizing the trait from spherical equivalent to myopia or hyperopia in each population did reduce sample size for each population compared to the number of individuals with measurements of spherical equivalent. This consequent reduction in power was the reason we added additional populations to our discovery meta-analysis compared to our refractive error meta-analysis[17], to offset the lower sample size. This current study is still, however, smaller than the Kiefer et al[18] study we were attempting to replicate and so some of the other loci may yet replicate in a larger study.

In summary, we have provided evidence in favor of replication of 11 loci involved in causation of myopia. Twelve loci that have been shown to be associated with myopia age at onset[18] showed "replication-level" association with hyperopia here (7 of these loci also showed replication-level association with the myopia trait; 5 loci only showed this level of association with hyperopia). Further research is required to determine whether any of the candidate genes identified near these associated SNPs are truly causing the development of refractive errors, or whether the actual causal variant is located in another nearby gene or other functional locus in high LD with the SNPs associated with the trait. Evidence for expression of many of these genes have indicated that they are active in the eye[19] and investigation of the ENCODE data suggests many loci have regulatory functions, which is consistent with the current hypothesis of regulation of eye growth through a visually-evoked signaling cascade. However, more research using in vitro and in vivo models is necessary to elucidate the underlying mechanisms of normal emmetropization and how it can be disrupted to produce refractive errors.

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3.5

Nine loci for ocular axial length identified through genome-wide association studies, including shared loci with refractive error

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ABSTRACT

Refractive errors are common eye disorders of public health importance worldwide. Ocular axial length (AL) is the major determinant of refraction and thus of myopia and hyperopia. We conducted a meta-analysis of genome-wide association studies for AL, combining 12,531 Europeans and 8,216 Asians. We identified eight genome-wide significant loci for AL, including *RSPO1, C3orf26, LAMA2, GJD2*, *ZNRF3*, *CD55*, *MIP* and *ALPPL2*, and confirmed one previously reported AL locus, *ZC3H11B*. Of the nine loci, five, *LAMA2, GJD2, CD55, ALPPL2* and *ZC3H11B,* were associated with refraction in 18 independent cohorts (n = 23,591). Differential gene expression was observed for these loci in minus-lens induced myopia mouse experiments and human ocular tissues. Two of the AL genes, *RSPO1* and *ZNRF3,* are involved in Wnt signaling, a pathway playing a major role in the regulation of eyeball size. This study provides evidence of shared genes between AL and refraction, but importantly, also suggests that these traits may have unique pathways.

INTRODUCTION

Myopia (nearsightedness), the most common form of refractive errors, is an ocular disorder of major public health importance worldwide, particularly in Asia. About 40% of adults and 80–90% of children completing high school are myopic in urban areas in East Asian countries, and 10–20% of them have high myopia.[1,2] Uncorrected myopia and refractive errors are leading causes of visual impairment.[3-6] Furthermore, adults with high myopia are at a substantially higher risk of potentially blinding pathologies, including glaucoma, retinal detachment and myopic maculopathy.[7] The correction of myopia and refractive errors in general by spectacles, contact lenses or refractive surgery can entail substantial socioeconomic costs[8,9] and does not treat the underlying mechanism of disease.

Myopia develops primarily from an eye that is excessively elongated axially and thus ocular axial length (AL) is an attractive endophenotype to investigate for several reasons. First, AL alone accounts for more than 40% of variation in refractive errors.[10-12] Magnetic resonance imaging studies of the orbit have also demonstrated that extremely high myopic eyes are generally prolate in shape with unusually long ALs, leading to associated visually disabling complications such as posterior staphylomas.[13,14] Second, the heritability of AL (67% to 94%) is consistently higher than that for refraction.[15-18] Furthermore, the measurement of AL (in mm) is more objective, precise and reproducible compared to assessments of refractive status.

While more than 30 myopia loci have been implicated in previous linkage and genome-wide association studies (GWAS), there have been few reports of AL-specific loci. A recent GWAS identified an association at *ZC3H11B* for both AL and high myopia in Asians.[19] To identify additional genetic variants that modulate AL, we conducted the largest international GWAS meta-analysis of AL to date in cohorts participating in the Consortium for Refractive Error and Myopia (CREAM).[20,21]

MATERIAL AND METHODS

We used a three-stage approach.[21] First, we performed a GWAS meta-analysis in 12,531 European ancestry individuals (stage 1). Second, we tested the cross-ethnic transferability of the associations from this first stage in 8,216 Asian ancestry individuals (stage 2). Lastly, we conducted a meta-analysis combining individuals of European and Asian ancestry, totaling 20,747 individuals (stage 3). We subsequently examined the effect of the associated AL loci on spherical equivalent (SE) in 23,591 individuals from 18 other independent cohorts.

Study populations in CREAM

All studies participating in this meta-analysis are part of the CREAM.[20,21] The discovery cohorts included 12,531 European ancestry individuals from 18 studies (Table 1), including ALSPAC Children,[22] BATS/TEST,[23] BMES,[24,25] Croatia-Korcula, Croatia -Split, Croatia-Vis,[26] ERF,[27,28] RS-I, RS-II, RS-III,[29] ORCADES,[30] and RAINE.[31-33] In addition, 8,216 Asian ancestry individuals from six cohorts (Table 1), including BES,[34] SCES,[35] SCORM,[36] SiMES,[37] SINDI,[35] and STARS Parents,[38] were included in the replication stage. General methods, demographics and phenotyping of the study cohorts have previously been described extensively and are provided briefly in Table 1. All

Table 1. Study cohorts and summary of axial length measures

Ethnicity	N	Study	Mean age (SD), yrs	Men, %	Axial length Mean (SD), mm	Range, mm	Methods of measurement
European	2069	ALSPAC Children	15.5 (0.3)	46.5	23.41 (0.87)	20.49, 26.57	IOLmaster
	1316	BATS/TEST	24.6 (11.9)	43.2	23.25 (0.87)	20.03, 28.25	IOLmaster
	1030	BMES	73.8 (7.8)	59.5	23.45 (1.04)	19.94, 29.86	IOLmaster
	826	Croatia-Korcula	55.8 (13.4)	35.1	23.19 (1.06)	18.55, 28.24	Echoscan US-1800
	352	Croatia-Split	50.0 (14.2)	44.3	23.39 (0.90)	20.98, 27.3	Echoscan US-1800
	552	Croatia-Vis	56.0 (14.0)	39.7	23.08 (0.90)	20.09, 26.48	Echoscan US-1800
	2397	ERF4	48.7 (14.2)	55.5	23.22 (1.04)	19.79, 27.30	A scan
	503	ORCADES	57.6 (13.7)	43.3	23.70 (1.08)	20.69, 28.00	IOLmaster
	1011	Raine	20.1 (0.4)	51.6	23.56 (0.89)	20.36, 27.94	IOLmaster
	676	RS-I	78.4 (4.4)	49.0	23.52 (1.06)	20.44, 27.72	Lenstar LS900
	1085	RS-II	72.0 (4.7)	47.2	23.50 (1.14)	19.87, 28.00	Lenstar LS900
	714	RS-III	59.3 (5.8)	42.6	23.56 (1.27)	19.79, 28.45	Lenstar LS900 and A scan
Asian	564	BES	62.05 (8.4)	35.5	23.07 (1.15)	19.90, 30.36	Lenstar LS900
	1720	SCES	57.6 (9.0)	51.7	23.95 (1.31)	20.87, 32.66	IOLmaster
	926	SCORM	10.8 (0.8)	51.7	24.13 (1.12)	21.05, 28.20	Echoscan US-800
	2141	SiMES	57.6 (10.7)	49.3	23.57 (1.04)	20.48, 31.11	IOLmaster
	2120	SINDI	55.9 (8.8)	51.4	23.41 (1.08)	19.07, 31.59	IOLmaster
	745	STARS Parents	38.8 (5.3)	51.0	24.64 (1.51)	21.66, 31.57	IOLmaster

ALSPAC, Avon Longitudinal Study of Parents and Children; BATS, Brisbane Adolescent Twins Study; TEST, Twins Eye Study in Tasmania; BMES, Blue Mountains Eye Study; ERF, Erasmus Rucphen Family Study; ORCADES, Orkney Complex Disease Study; RS, Rotterdam Study; BES, Beijing Eye Study; SCES, Singapore Chinese Eye Study Singapore; SCORM, Singapore Cohort Study of the Risk Factors for Myopia; SiMES, Singapore Malay Eye Study; SINDI, Singapore Indian Eye Study; STARS, Strabismus, Amblyopia, and Refractive Error Study of Preschool Children; SD, standard deviation.

studies were performed with the approval of their local Medical Ethics Committee, and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Independent populations in CREAM

To examine whether the loci affecting AL contributed to SE, we studies associations with SE in an additional 18 studies (Table S1), including the 1958 British Birth Cohort,[39] ALSPAC Mothers,[40] ANZRAG,[41] AREDS 1a1b, AREDS 1c,[15,16] DCCT,[42] EGCUT,[43] FECD,[44] FES,[45] FITSA,[46] GHS 1, GHS 2, KORA,[47-50] OGP Talana,[51] SP2,[52] TwinsUK,[53] WESDR,[54] and Young Finns Study.[55] Only SE but not AL measures were available in these additional 18 CREAM studies. Detailed study design and methodology of these studies have been published elsewhere. Descriptive data on demographics and phenotypes of these cohorts are briefly shown in Table S1**.**

Phenotype measurements

All studies used a similar protocol for ocular phenotype measurements. Eligible participants underwent an ophthalmologic examination including measurements of AL and refraction of both eyes. AL was measured using either optical laser interferometry or A-scan ultrasound biometry (Table 1). Refraction was measured by autorefractor and/or subjective refraction (Table S1). SE was calculated according to the standard formula (SE = sphere+1⁄2 cylinder).

Genotyping and imputation

The study samples were genotyped on either the Illumina (San Diego, CA, USA) or Affymetrix (Santa Clara, CA, USA) platforms. Each study performed single nucleotide polymorphism (SNP) imputation using the genotype data, together with the HapMap Phase II ethnically matched reference panels (CEU, JPT+CHB, or the 4 HapMap populations) on the basis of build 36 databases (release 22 or 24). The Markov Chain Haplotyping software, IMPUTE[56,57] or MACH,[58] were adopted for imputation. A detailed description regarding genotyping platforms and imputation procedures for each study is provided on Tables S2 and S3.

Stringent quality control of genotype data was applied in each cohort. Samples with low call rates (<95%) or with gender discrepancies were excluded. Cryptically related samples and outliers in population structure from principal component analyses were also excluded. SNPs flagged with missingness >5%, gross departure from Hardy Weinberg Equilibrium (P value $<10^{-6}$, except in the ALSPAC study where a threshold of $<10^{-7}$ was used) and minor allele frequency (MAF) <1% were removed from further analyses.

Statistical Analysis

For each study, an allele-dosage regression model at each genotyped or imputed SNP was conducted to determine its association with AL as a quantitative trait as well as its association with SE. Individuals with prior refractive or cataract surgery, or other intra-ocular procedures that could alter refraction, were excluded. The mean of the right and left eyes was taken. When data from only one eye were available, the AL or SE of this eye was used. Sample outliers with AL value exceeding 4 standard deviations from the mean were excluded at the study level. We assumed an additive genetic model where the dosage of each SNP is a continuous variable ranging from

0 to 2 for minor alleles carried. Primary analysis for AL was adjusted for age, sex and height (as height was consistently correlated with AL[59,60]) and in the case of SE for age and sex. Additional adjustment for principal components was carried out according to the population substructure in each individual study.

The per-SNP meta-analyses were performed using METAL software with weighted inverse-variance approach, assuming fixed effects, as for initial discovery purposes, the fixed-effects model is preferred for increased statistical power.[61] A Cochran's Q test was used to assess heterogeneity across studies.[62] Imputation quality scores were reviewed for the top SNPs reported to ensure good imputation quality (proper-info of IMPUTE or R^2 of MACH >0.3).

Gene-based testing was conducted using VEGAS software[63] on the European ancestry and Asian ancestry meta-analysis results separately. VEGAS incorporates information from the full set of markers within a gene and thus can be more powerful than tests of individual SNPs if there are multiple risk variants within a gene. VEGAS corrects for LD and gene size by conducting simulations based on the LD structure in the population of interest (here, European or Asian ancestry). VEGAS was hence run separately on all the European and Asian GWAS data, with results for each gene combined at the end using meta-analysis on the two sets of gene-based *P*-values using Fisher's methods. For samples of European descent, we used the HapMap 2 CEU population as the reference to estimate patterns of LD. For Asian ancestry groups, we used the combined HapMap 2 JPT and CHB populations as the reference population to approximate linkage disequilibrium (LD) patterns. To include gene regulatory regions, SNPs were included if they fell within 50 kb of a gene.

VEGAS-Pathway analysis[63,64] was carried out using prespecified pathways from Gene Ontology. Pathways of with 10 to 1,000 components were selected, yielding 4,628 pathways. Pathway analysis was based on combining gene-based test results from VEGAS. Pathway *P*-values were computed by summing χ^2 test statistics derived from VEGAS *P*-values. Empirical VEGAS-Pathway P values for each pathway were computed by comparing the summed χ^2 test statistics from real data with those generated in 500,000 simulations where the relevant number (according to the size of the pathway) of randomly drawn χ^2 test statistics was summed. To ensure that clusters of genes did not adversely affect results, within each pathway, gene sets were pruned such that each gene was >500 kb away from all other genes in the pathway. Where required, all but one of the clustered genes was dropped at random when genes were clustered. We performed meta-analysis on the two sets of pathway *P*-values using Fisher's method.

Differential gene expression in a mouse model of myopia

Animal study approval was obtained from the SingHealth Institutional Animal Care and Use Committee (AAALAC accredited). All procedures performed in this study complied with the Association of Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmology and Vision Research. Experimental myopia was induced in B6 wild-type (WT) mice (n = 36) by applying a -15.0 diopter spectacle lens on the right eye (experimental eye) for 6 weeks from post-natal day 10. The left uncovered eye served as the contra-lateral control eye. Age-matched naive mice eyes were also used as independent control eyes (n = 36).[65,66] Eye biometry,

refraction, tissue collection, RNA extraction, real-time polymerase chain reaction (PCR) qRT-PCR methods and analysis were followed as described previously.[19] qRT-PCR primers (Table S4) were designed using ProbeFinder 2.45 (Roche Applied Science, Indianapolis, IN) and performed using a Lightcycler 480 Probe Master (Roche Applied Science, Indianapolis, IN). The experiments were repeated in triplicate. Gene expression of all identified genes in the control and experimental groups was quantified using the $2^{-\Delta\Delta Ct}$ method.[67] Student's *t*-test was performed to determine the significance of the relative fold difference of mRNA between the myopic eyes of the experimental mice and the age-matched controls.

Gene expression in human tissues

Adult ocular samples were obtained from normal eyes of an 82-year-old European ancestry female from the North Carolina Eye Bank, Winston-Salem, North Carolina, USA. All adult ocular samples were stored in Qiagen's RNA*later* within 6.5 hours of collection and shipped on dry ice overnight to the lab. Isolated tissues were snap-frozen and stored at -280 °C until RNA extraction. RNA was extracted from each tissue sample independently using the Ambion *mir*Vana total RNA extraction kit. The tissue samples were homogenized in Ambion lysis buffer using an Omni Bead Ruptor Tissue Homogenizer per protocol. Reverse transcription reactions were performed with Invitrogen SuperScript III First- Strand Synthesis kit. The expression of the identified genes was assessed by running 10 µl reactions with Qiagen's PCR products consisting of 1.26 µl H2O, 1.0 µl 10X buffer, 1.0 µl dNTPs, 0.3 µl MgCl, 2.0 µl Q-Solution, 0.06 µl taq polymerase, 1.0 µl forward primer, 1.0 µl reverse primer and 1.5.0 µl cDNA. The reactions were run on a Eppendorf Mastercycler Pro S thermocycler with touchdown PCR ramping down 1°C per cycle from 72 °C to 55 °C followed by 50 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds with a final elongation of 7 minutes at 72 °C. All primer sets were designed using Primer3.[68] Products were run on a 2% agarose gel at 70 volts for 35 minutes. Primer sets were run on a custom tissue panel including Clontech's Human MTC Panel I, Fetal MTC Panel I and an ocular tissue panel.

RESULTS

We analyzed 2.5 million genotyped and imputed SNPs (Table S2). The genomic control inflation factor (λ) for individual studies (Table S2) as well as for the meta-analysis (λ_{GC} = 1.06) and quantile-quantile plots (Figure S1) showed little evidence for inflation.

Per-SNP meta-analysis

In the first stage, a total of 177 SNPs, representing 24 physically distinct loci, were associated with $P < 1 \times 10^{-5}$ in the European ancestry discovery cohort (Table S5). Of them, we identified one locus at chromosome 15q14 in the proximity of *GJD2* ([OMIM #607058] rs11073058, $P = 2.0 \times 10^{-8}$) exceeding genome-wide significance level ($P < 5 \times 10^{-8}$; Table 2), which was previously reported to be associated with refractive errors.[69] We took the 177 SNPs forward for replication in the Asian cohorts (stage 2). Five regions showed significant evidence of replication ($1.12 \times 10^{-9} \leq P \leq 1.18 \times 10^{-2}$, Table 2), including *RSPO1* (OMIM #609595), *C3orf26* and *LAMA2* (OMIM #156225), and regions close to *ZC3H11B* and *GJD2.* In the combined meta-analysis of all 18 European and Asian cohorts (stage 3, n = 20,747), all five loci surpassed genome-wide significance level (3.97×10^{-13}

≤1

P

≤1.24

Table 2. Associations with ocular length in the European ancestry cohorts with results in the Asian cohorts and combined analysis

				European ancestry cohorts (Stage1, n = 12531)				**Asian cohorts (Stage 2, n = 8216)**				**Combined (Stage 3, n = 20747)**				
Lead SNP[c]	Chr	Position[a]	EA	EAF	Beta[b]	SEM	***P* value**	EAF	Beta[b]	SEM	***P* value**	**EAF**	**Beta[b]**	**SEM**	***P* value**	**Localization relative to protein-coding genes[c]**
rs4074961	1	37865310	T	0.43	0.06	0.01	$6.6x10^{-6}$	0.45	0.10	0.02	$1.1x10^{-9}$	0.44	0.07	0.01	$4.0x10^{-13}$	Intron 4 of *RSPO1* (OMIM #609595)
rs994767	1	217842055	A	0.45	-0.06	0.01	$1.2x10^{-6}$	0.32	-0.10	0.02	$4.4x10^{-7}$	0.41	-0.07	0.01	$9.6x10^{-12}$	7kb upstream of *ZC3H11B*
rs9811920	3	101326983	A	0.41	0.07	0.01	$3.0x10^{-7}$	0.36	0.13	0.03	$6.0x10^{-6}$	0.40	0.08	0.01	$4.9x10^{-11}$	Intron 1 of *C3orf26*
rs12193446	6	129861731	A	0.91	0.12	0.02	$1.1x10^{-7}$	0.98	0.28	0.11	$1.2x10^{-2}$	0.91	0.12	0.02	$1.2x10^{-8}$	Intron 58 of *LAMA2* (OMIM #156225)
rs11073058	15	32776918	T	0.43	0.07	0.01	$2.0x10^{-8}$	0.50	0.06	0.02	$4.7x10^{-4}$	0.45	0.07	0.01	$4.3x10^{-11}$	57kb upstream of *GJD2* (OMIM #607058)
Additional locus identified through the combined analysis of European and Asian cohorts																
rs12321	22	27783183	C	0.44	-0.05	0.01	$1.1x10^{-5}$	0.49	-0.06	0.02	$9.9x10^{-4}$	0.46	-0.05	0.01	$4.1x10^{-8}$	3' UTR of *ZNRF3* (OMIM #612062)

SNPs with $P < 1x10^{-5}$ in European ancestry cohorts were brought for replication in Asians. Genome-wide significance is defined as $P < 5.0x10^{-8}$.

[a]Position is based on NCBI human genome build 36. [b]Effect sizes on axial length are in mm. [c]Lead SNPs of each locus identified in the combined meta-analysis (stage 3) are presented. The lead SNPs in the combined meta-analysis are the same as the lead SNPs in the European-only analysis (stage 1) for all loci, except for the 1q41 locus near *ZC3H11B*, where the lead SNP in European-only analysis is rs10863469 (position, 217844091; frequency of effect allele T = 0.47; Beta = 0.47, $P = 1.2 \times 10^{-6}$), being in high LD ($r^2 = 0.84$) with rs994767. SNP, single nucleotide polymorphism; Chr, chromosome; EA, effect allele; EAF, effect allele frequency, SEM, standard error of the mean.

x 10^{-8}; Table 2 and Figure 1). Furthermore, in stage 3 we detected an additional genome-wide significant locus at *ZNRF3* (OMIM #612062, $P = 4.08 \times 10^{-8}$, Table 2).

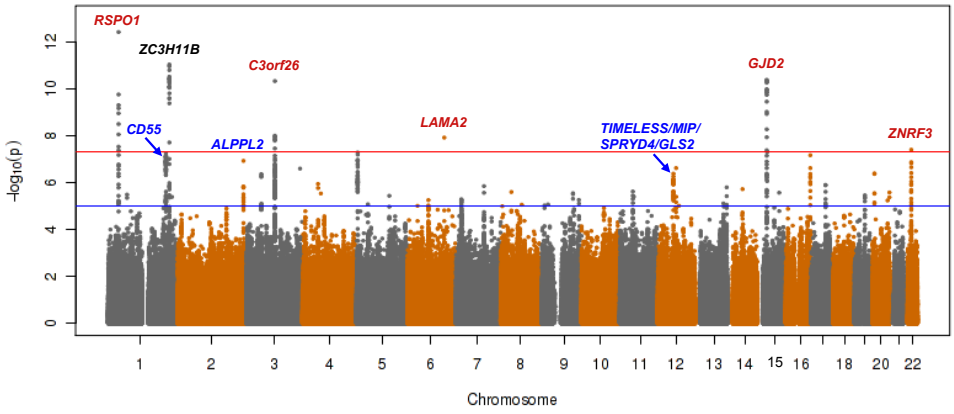

Figure 1. Summary of meta-analysis results for genome-wide association to ocular axial length. Data of both directly genotyped and imputed SNPs are presented in the Manhattan plot. The *y* axis represents $-\log_{10} P$ values for association with axial length, and the *x* axis represents chromosomes and base-pair positions based on human genome build 36. The horizontal red line indicates the genome-wide significance level of $P < 5.0 \times 10^{-8}$. The horizontal blue line indicates the suggestive significance level of $P < 1.0 \times 10^{-5}$. The previously described locus for axial length is labeled in black. Other loci reaching genome-wide significance identified from the per-SNP meta-analysis are labeled in red. The genes identified in gene-based tests are labeled in blue.

Overall, the significant regions included six loci for AL, including *RSPO1, C3orf26, LAMA2, GJD2, ZNRF3*, and one previously identified locus for AL at 1q41 close to *ZC3H11B*.[19] A common SNP in *RSPO1* displayed the strongest evidence for association (rs4074961, β = 0.07 mm per copy of risk allele, $P = 3.97 \times 10^{-13}$), with no evidence of heterogeneity ($I^2 = 0\%$, $P = 0.78$) across the 18 AL cohorts (Table S6), while the strongest effect was observed for the rarer intronic variant in *LAMA2* (rs12193446, β = 0.12 mm, $P = 1.24 \times 10^{-8}$). Figure 2 shows the regional association plots for the six loci significant in single SNP tests. Forest plots showing the effect sizes across cohorts are provided in Figure S2. We constructed a multi-locus genetic risk score to evaluate the combined effects of the AL SNPs in the Blue Mountains Eye Study[24,25] and the Singapore Chinese Eye Study,[35] both of which were part of the 18 AL discovery cohorts. Figure S3 shows that the odd ratios for longer AL (Tertile 3 vs. Tertile 1) were higher with increasing genetic risk scores.

Gene-based meta-analysis

In addition to per-SNP meta-analysis, we applied gene-based tests using VEGAS,[25] with genome-wide significance declared if $P_{\text{gene-based}} < 0.05/17872 = 2.8 \times 10^{-6}$ (17,872 genes tested). Over and above the loci found in per-SNP tests, three additional genomic regions were genome-wide significantly associated with AL using gene-based tests (Table 3): *CD55* (OMIM #125240), *ALPPL2* (OMIM #171810) and *TIMELESS*/*MIP*/*SPRYD4*/*GLS2* (OMIM #603887 for *TIMELESS*). Figure S4 shows the regional association for the three loci significant in gene-based tests.

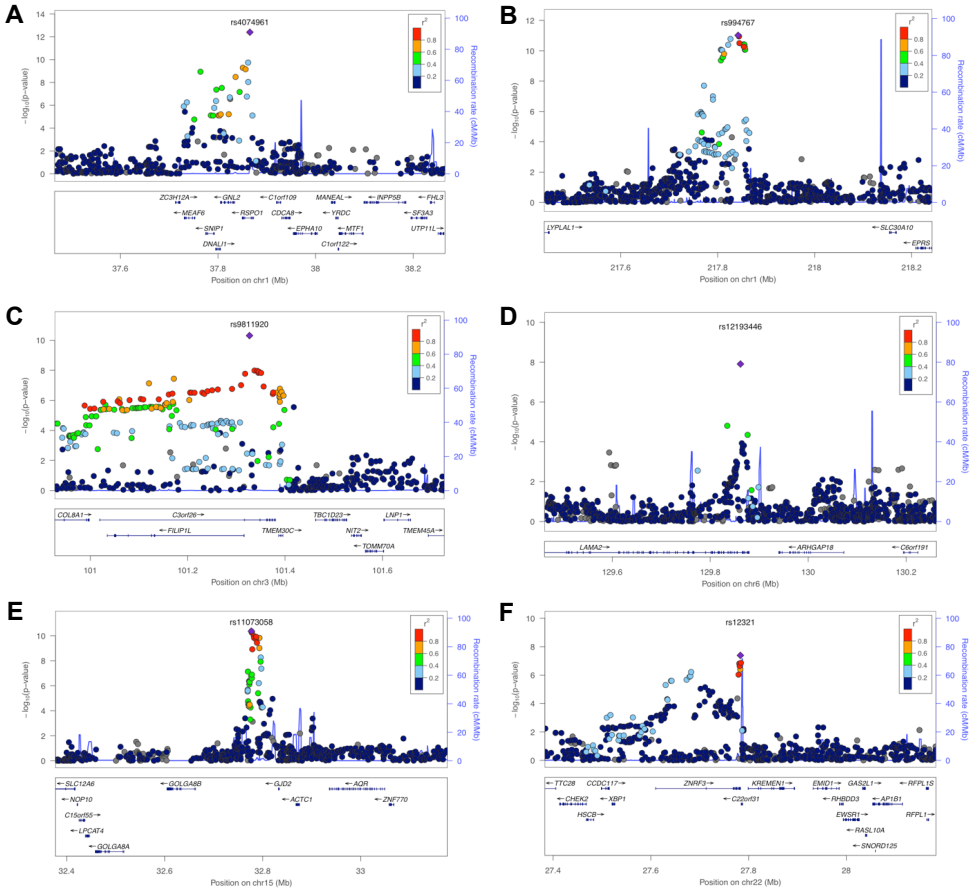

Figure 2. Regional association plots and recombination rates of the loci associated with ocular axial length.

Data are shown for association at chromosome **A**. 1p34.3 (*RSPO1*), **B**. 1q41 (*ZC3H11B*), **C**. 3q12.1 (*C3orf26*), **D**. 6q22.33 (*LAMA2*), **E**. 15q14 (*GJD2*), and **F**. 22q12.1 (*ZNRF3*) in the combined meta-analysis. Data of both directly genotyped and imputed SNPs are presented. In each panel, the genotyped SNP with the most significant association is denoted with a purple diamond. The color coding of all other SNPs indicates LD with the lead SNP, estimated by CEU r^2 from phase II HapMap: red, $r^2 \geq 0.8$; yellow, $0.6 \leq r^2 < 0.8$; green, $0.4 \leq r^2 < 0.6$; cyan, $0.2 \leq r^2 < 0.4$; blue, $r^2 < 0.2$; and gray, r^2 unknown. The left *y* axis represents $-\log_{10}$ *P* values for association with axial length, the right *y* axis represents the recombination rate, estimated from the International HapMap Project, and the *x* axis represents base-pair positions along the chromosome based on human genome build 36. Gene annotations are taken from the University of California Santa Cruz (UCSC) genome browser. The plots were created using LocusZoom.

Association with refraction

We subsequently assessed the association of these AL SNPs and genes with SE in 23,591 individuals from 18 independent studies in CREAM that had SE but no AL measures (Tables S1 and S3). We found associations (*P* <0.05) with SE for three of the six AL SNPs (Table 4 and

Table 3. Loci associated with ocular axial length in gene-based tests

Gene	OMIM #	Chr	Start position[a]	End position[a]	$P_{gene\text{-}based}$ value European ancestry cohorts	Asian cohorts	Combined[b]
CD55	125240	1	205561439	205600934	1.3×10^{-5}	9.6×10^{-4}	2.3×10^{-7}
ALPPL2	171810	2	232979795	232983669	6.4×10^{-5}	1.7×10^{-3}	1.8×10^{-6}
TIMELESS/MIP/ SPRYD4/GLS2[c]	603887	12	55097173	55168448	2.0×10^{-7}	7.3×10^{-2}	2.8×10^{-7}

[a]Position is based on NCBI human genome build 36. Note this is the start and stop position of the gene. For gene-based tests, 50kb was added to either side to account for possible regulatory variants which fall outside the gene boundaries.
[b]Gene-based genome-wide significance was defined as $P < 2.80 \times 10^{-6}$. Only loci that were genome-wide significant in gene-based testing but not genome-wide significant in per SNP testing are shown.
[c]*TIMELESS* was the most significant gene in the region. Due to the +/- 50kb added to the definition for each gene and the close proximity of the genes, *MIP, SPRYD4, GLS2* and *TIMELESS* all had similar gene-based *P*-values (ranged from 1.4×10^{-6} to 2.8×10^{-7} for the combined analysis), and thus only *P*-value and OMIM # for *TIMELESS* is presented.
Chr, chromosome.

Table 4. Association with spherical equivalent of the SNPs most strongly associated with axial length in each genomic locus in independent cohorts

Lead SNP	Nearest gene	Effect allele	Beta[a]	SEM	***P* value**
rs4074961	*RSPO1* (OMIM #609595)	T	0.004	0.023	0.84
rs994767	*ZC3H11B*	A	0.054	0.022	1.3×10^{-2}
rs9811920	C3orf26	A	-0.022	0.022	0.31
rs12193446	*LAMA2* (OMIM #156225)	A	-0.242	0.039	3.6×10^{-10}
rs11073058	*GJD2* (OMIM #607058)	T	-0.121	0.022	1.7×10^{-8}
rs12321	*ZNRF3* (OMIM #612062)	C	-0.004	0.021	0.86

[a]Effect sizes on spherical equivalent are in diopters.
SNP, single nucleotide polymorphism; SEM, standard error of the mean.

Table 5. Association of the axial length genes identified in gene-based tests with spherical equivalent in independent cohorts

Gene	OMIM #	**Chr**	$P_{gene\text{-}based}$ **value**[a]
CD55	125240	1	4.5×10^{-6}
ALPPL2	171810	2	8.3×10^{-3}
TIMELESS/MIP/SPRYD4/GLS2	603887	12	0.14

[a]The association with spherical equivalent was assessed in 17 European ancestry cohorts of the 18 independent cohorts, using the HapMap 2 CEU population as the reference to estimate patterns of LD.
[c]Due to the +/- 50kb added to the definition for each gene and the close proximity of the genes, *MIP, SPRYD4, GLS2* and *TIMELESS* all had similar gene-based *P*-values (ranged from 0.14 to 0.20 for the combined analysis), and thus only *P*-value and OMIM # for *TIMELESS* is presented.
Chr, chromosome.

Figure S5), including rs994767 (*ZC3H11B*, P = 0.013), rs11073058 (*GJD2*, P =1.66 x 10^{-8}), and rs12193446 (*LAMA2, P* = 3.58 x 10^{-10}), with directions of the SE association being consistent with AL. For example, the risk allele T of rs11073058 in *GJD2* was associated with both longer AL and more myopia (more negative SE). In gene-based tests, only *CD55* (P = 4.5 x 10^{-6}) and *ALPPL2 (P* = 8.3 x 10^{-3}*)* were associated with SE (Table 5).

SNPs close to *CD55* had reached genome-wide significant association with SE in the meta-analysis of all CREAM cohorts (i.e. with and without AL measures).[21] There was an association with SE at *CHRNG*, along with a less significant independent hit near *ALPPL2* (125kb away).[21] Our AL gene-based results showed a genome-wide significant signal at *ALPPL2* but not at *CHRNG*. There was also an association with SE at *RDH5*,[21] on the same chromosomal band as the AL signal at *MIP* (OMIM #154050), but *RDH5* and *MIP* are 727kb apart without LD between them, suggesting that they are independent signals.

Pathway analysis

We conducted pathway analysis using VEGAS-Pathway[63,64] by combining the gene-based *P*-values for 4,628 pre-specified pathways. The most significant pathway was the "Wnt receptor signaling" pathway (P = 2.9 x 10^{-5}). The Bonferroni corrected P value was 0.13 (for the total number of 4,628 pathways tested). However, Bonferroni correction is an over correction, as many of the pathways have overlapping genes. The identification of the Wnt signaling pathway, even if only nominally associated, is of interest as the pathway involves two genes identified from the per-SNP tests. Also among the top 10 pathways were "lens development in camera-type eye" (P = 2.4 x 10^{-4}) and "collagen" pathways (P = 5.1 x 10^{-4}, Table S7). The collagen pathway was implicated in a recent meta-analysis of corneal thickness.[64]

Gene expression

Differential expression of the nearest genes in the six implicated loci from per-SNP meta-analysis (Table S4) was assessed by measuring mRNA levels in minus-lens induced myopia mouse models.[65,66] The mRNA levels of all six genes had a two-fold difference in the induced myopic eyes as compared to the control eyes in most of the tissues tested: sclera, retinal pigment epithelium (RPE) and neural retina (Figure S6).

In human ocular tissue, we have previously shown that *ZC3H11B* is expressed in neural retina, RPE and sclera,[19] *LAMA2* is expressed in sclera and optic nerve, and *CD55* is expressed in retina, choroid and cornea, whereas *GJD2* is less abundant in sclera and other ocular tissues.[21] In this study, we measured the mRNA expression levels of the other genes in adult ocular tissues using reverse-transcriptase PCR. We found that *C3orf26*, *ZNRF3* and *TIMELESS* were expressed in most ocular tissues while the expression of *RSPO1*, *ALPPL2* and *MIP* was less strong and/or more restricted (Table S8).

DISCUSSION

We identified five AL loci (*RSPO1, C3orf26, LAMA2, GJD2* and *ZNRF3*) and confirmed the previously described locus (*ZC3H11B*) using per-SNP tests. In addition, three loci (*CD55*, *ALPPL2 and TIMELESS/MIP/SPRYD4/GLS2*) were identified using gene-based tests. Therefore, a total of nine AL loci were identified in this meta-analysis. Seven of the nine AL loci are located within the genomic region of protein-coding genes (Tables 2 and 3). Of note, two of them (*RSPO1* and *ZNRF3*) encode proteins that are directly involved in the Wnt signaling pathway. *RSPO1* is a member of a family of secreted proteins that act as stem-cell growth factors by enhancing the Wnt signaling pathway.[70] On the other hand, *ZNRF3* is a membrane-bound protein that acts as a negative regulator of the Wnt signaling pathway by mediating degradation of the Wnt receptor complex components Frizzled and *LRP6*.[71] The two proteins have recently been shown to interact, *RSPO1* enhancing Wnt signaling through inhibition of *ZNRF3*.[71] The Wnt signaling was the most significant pathway in our analysis, further supporting its prominent role in vertebrate eye development.[72] Indeed, overexpression of a dominant negative variant of human *ZNRF3* in zebrafish embryos induces small eye or loss of eyes.[71]

Remodeling of extracellular matrix in sclera plays an important role in changes of eye size during myopia development. *LAMA2* encodes the alpha 2 chain of laminin, a major extracellular protein of the basement membrane. We used HaploReg[73] to search for evidence of a functional role for variants at the *LAMA2* locus, as it has the largest per-allele effect on AL. The intronic lead SNP rs12193446 lies within the promoter and enhancer histone marks as well as DNase hypersensitive sites. Analysis with RegulomeDB2[74] suggested that rs12193446 occurs in a region that binds *EP300, TCF4, STAT3, GATA2* and *RFX4*. Four of these interactions (*EP300, TCF4, STAT3* and *GATA2*) were predicted by HaploReg[73] to be affected by the genotype at rs12193446. Mutations in the cognate gene for TCF4 cause Pitt-Hopkins syndrome (PTHS [OMIM #610954]), the predominant ocular feature of which is high-grade myopia.[75] Interestingly, common genetic variants in *TCF4* (OMIM #602272) have also been associated with Fuchs corneal dystrophy, suggesting the pleiotropic effects of *TCF4* on ocular diseases.[76]

Gene-based testing implicated the *TIMELESS/MIP/SPRYD4/GLS2* region although determining which of these genes are functionally relevant is difficult as there are multiple association signals in the region. *MIP* is an interesting candidate gene as it is expressed in the ocular lens and is required for correct lens function.[77] *CD55*, implicated here in AL and previously in SE,[21] is known to elevate cytosolic calcium ion concentration.

For all six of the genes identified in our per-SNP meta-analysis, we found evidence for differential expression in a mouse model of myopia. Differential expression was observed in the mouse sclera and retina as well as RPE cells, suggesting a role for these genes in myopia. Further strengthening our results, the expression data showed all but one of these genes expressed in the adult human eye. These data potentially provide insights into the complexity of AL elongation and myopia at the biological level. Some genes, namely *ZC3H11A*, *GJD2* and *LAMA2*, showed changes in expression that are consistently in the same direction across the different eye sections analyzed, whereas others, namely *RSPO1*, *C3orf26* and *ZNRF3*, showed variable directions of differential

expression. These results, together with the pathway analysis results, suggest that the genetic mechanisms of myopia are complex, involving more than one eye component.

We have previously shown that up to 50% of the variation in SE is due to shared genetic factors with AL.[78] Thus, we undertook further analyses and found that five of the nine AL loci are also associated with SE. Furthermore, we looked up the association of AL with the SNPs discovered from the recent CREAM GWAS meta-analysis on SE in 32 cohorts[21] and observed that 23 of the 29 SNPs identified with SE have significant effects on AL ($P < 0.05$, Table S9). This has important implications. First, it confirms the previous findings in twins[78] that there are common genetic determinants of the two traits, such as variants in *GJD2*, *LAMA2*, *CD55* and *ALPPL2*. Second, it indicates that some genetic variants for AL do not influence SE, suggesting they regulate the co-ordinated scaling of eye size.[79] For example, the SNP in *RSPO1* showed the strongest evidence of association with AL, yet it had no association with refractive error. In eyes without refractive error, AL and corneal curvature are carefully scaled relative to one another, and have a high phenotypic correlation between them.[80] Therefore, genes like *RSPO1,* might mediate a compensatory mechanism through changes in corneal curvature or optical power thus balancing their effects on SE.

Shorter axial length is a major risk factor for angle closure glaucoma. A recent GWAS on primary angle closure glaucoma identified three genome-wide significant loci located at *PLEKHA7* (OMIM #612686), *COL11A1* (OMIM #120280) and *PCMTD1-ST18*.[81] However, none of the common variants in the three loci were significantly associated with AL in our meta-analysis (Table S10). This suggests that susceptibility genes do not overlap between primary angle closure glaucoma and eyes with shorter axial length.

In summary, we identified nine loci associated with AL. They fall into two groups, one also influencing common refractive error variation, the other, which includes two genes in the Wnt signaling pathway, uniquely determining eye size with little effect on natural refractive status. Further elucidation and characterization of the causal variants underlying the growth of ocular component dimensions and the development of myopia may enable new pathway and target identification, leading to potential prevention and treatment development.

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Genome-wide association study for refractive astigmatism reveals genetic co-determination with spherical equivalent and refractive error: the CREAM consortium

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ABSTRACT

To identify genetic variants associated with refractive astigmatism in the general population, meta-analyses of genome-wide association studies were performed for: White Europeans aged at least 25 years (20 cohorts, n=31,968); Asian subjects aged at least 25 years (7 cohorts, n=9,295); White Europeans aged <25 years (4 cohorts, n=5,640); and all independent individuals from the above three samples combined with a sample of Chinese subjects aged <25 years (n=45,931). Participants were classified as *cases* with refractive astigmatism if the average cylinder power in their two eyes was at least 1.00 diopter and as *controls* otherwise. Genome-wide association analysis was carried out for each cohort separately using logistic regression. Meta-analysis was conducted using a fixed effects model. In the older European group the most strongly associated marker was downstream of the neurexin-1 (*NRXN1*) gene (rs1401327, *P*=3.92E-8). No other region reached genome-wide significance, and association signals were lower for the younger European group and Asian group. In the meta-analysis of all cohorts, no marker reached genome-wide significance: The most strongly associated regions were, *NRXN1* (rs1401327, *P*=2.93E-07), *TOX* (rs7823467, *P*=3.47E-07) and *LINC00340* (rs12212674, *P*=1.49E-06). For 34 markers identified in prior GWAS for spherical equivalent refractive error, the beta coefficients for genotype versus spherical equivalent, and genotype versus refractive astigmatism, were highly correlated (r=-0.59, *P*=2.10E-04). This work revealed no consistent or strong genetic signals for refractive astigmatism, however the *TOX* gene region previously identified in GWAS for spherical equivalent refractive error was the second most strongly associated region. Analysis of additional markers provided evidence supporting widespread genetic co-susceptibility for spherical and astigmatic refractive errors.

INTRODUCTION

Refractive astigmatism results from the optical summation of the eye's corneal astigmatism and astigmatism from internal eye components (e.g., lens). In most individuals, these two sources of astigmatism tend to compensate for each other, such that overall refractive astigmatism is typically low in magnitude[1]. High levels of refractive astigmatism are usually the result of high corneal astigmatism rather than high internal astigmatism[2,3]. Astigmatism in infancy is a risk factor for amblyopia[4]. In later life, astigmatism commonly accompanies myopia and hyperopia[5-7], reducing visual acuity unless corrected by spectacles, contact lenses or refractive surgery[8].

The results of twin[9-12], family[13,14] and molecular genetic studies[15-17] suggest that astigmatism is highly heritable, as does its high prevalence in specific ethnic groups such as Native Americans[18-20]. For refractive astigmatism, the heritability has been estimated at 0.33 to 0.63 from twin studies[10,21,22]. Using a case-control genome-wide association study (GWAS) meta-analysis of 8,513 individuals of Asian ethnicity, Fan et al.[15] identified the *PDGFRA* gene on chromosome 4q12 as a susceptibility locus for corneal astigmatism. Cases were defined as subjects with corneal astigmatism (averaged between the two eyes) of at least 0.75 dioptres (D) and controls as those with corneal astigmatism less than 0.75 D. Three single nucleotide polymorphisms (SNPs) attained genome-wide significance (P <5.0E-08); rs7677751, rs2307049 and rs7660560. SNPs in the same region of *PDGFRA* have since been found to be associated with both corneal curvature and axial length[23-25], but not with spherical refractive error[24]. A second GWAS meta-analysis in 22,100 individuals of European descent by Lopes et al.[16] reported suggestive evidence that SNPs in the *VAX2* gene on chromosome 2p13 also confer susceptibility to refractive astigmatism (most strongly associated SNP, rs3771395; P = 2.0E-07). These authors modelled astigmatism as a continuous trait, by using an inverse normal transformation of the refractive astigmatism averaged between the two eyes.

The GWAS meta-analyses of Fan et al.[15] and Lopes et al.[16] both assessed large numbers of individuals derived from cohorts that were largely population-based. It is therefore unlikely that common autosomal genetic variants, i.e. those with a minor allele frequency (MAF) > 5%, with profound effects on the risk of developing astigmatism (e.g. OR >2) exist, as both studies would have had high power to detect them. Instead, the results of the two studies imply that most of the additive genetic risk for astigmatism arises from the combined action of a large number of individual risk variants, each with a small effect. This scenario, which also holds for spherical refractive error[26-29], suggests that substantially increasing the sample size of GWAS meta-analyses will be an effective method of discovering new variants, albeit with increasingly diminishing returns[30]. Here, we describe the largest GWAS for refractive astigmatism yet undertaken involving almost 46,000 persons.

MATERIAL AND METHODS

Selection of studies for inclusion in the meta-analysis

The CREAM consortium comprises researchers from more than 30 research groups who share a common interest in the genetics of refractive error. From March to July 2012, all Principal Investigators (PI's) of studies known to CREAM members who had collected refractive error

phenotype information and genome-wide genotyping information on a study sample were invited to join CREAM. An analysis plan detailing the protocol for the astigmatism GWAS meta-analysis was circulated, inviting all PI's to perform the requested analyses and to submit GWAS results for their study sample. There were no restrictions on which studies were eligible to join the meta-analysis.

Study cohorts and meta-analysis overview

GWAS results were meta-analyzed for a total of 32 cohorts. The subject demographics of the cohorts are summarized in Table 1: Further details can be found in the Supplement and in previous publications[31-58]. The mean age of the participants in each cohort varied from 15 to 74 years and 37,608 of them were of White European ancestry while 10,212 were of Asian ancestry. Because the magnitude and axis of astigmatism is known to vary with age[59,60], and to limit the effects of differing SNP-causal variant relationships across ethnicities, meta-analyses were carried out separately for (a) White Europeans aged < 25 years, (b) White Europeans aged ≥ 25 years, and (c) Asians aged ≥ 25 years. This age classification scheme follows that adopted previously by the CREAM consortium[28,61], and was agreed to by the CREAM Executive Committee prior to commencement of the meta-analyses. A final meta-analysis was performed combining all independent samples from these three groups with the SCORM study of Asians aged <25 years. Each participating study defined the astigmatism trait in the same manner and performed association analyses specifically for this study using equivalent logistic regression models (described below and in the supplement).

Phenotypic assessment

Subjects underwent an ophthalmic examination that included either subjective refraction, cycloplegic autorefraction or non-cycloplegic autorefraction (Supplemental Methods and Supplemental Table S1a). Astigmatism was defined in the same way during association analysis in all cohorts participating in this meta-analysis study. Participants with conditions that could alter refraction, such as cataract surgery, laser refractive procedures, retinal detachment surgery, keratoconus or ocular or systemic syndromes were excluded. Additional exclusion criteria were, firstly, a cylinder power ≥ 5.00 D in either eye (to exclude subjects with undiagnosed keratoconus or potential measurement errors), and secondly, a difference in cylinder power between the two eyes beyond four standard deviations from the mean (except for subjects with data for only one eye). Subjects were classified as astigmatic *cases* if the average cylinder power in the two eyes was ≥ 1.00 D and as *controls* otherwise (note that cylinder axis was ignored). The threshold value of 1.00 D was chosen due to its common usage in prior work[8,62]. The average of the two eyes was taken in order to maximise statistical power[63].

Genotyping and genotype imputation

Genotyping and imputation were carried out as described previously[28]. Briefly, participants in each cohort were genotyped using a whole genome SNP platform. The genotypes of subjects that passed a series of quality control (QC) filters, including call rate at least >95% and ancestry matching that of the reference population, were imputed to a common set of markers (HapMap

Table 1. Cohort demographics

Study	Ethnicity	N (Cases/controls)	Age (mean ± SD) years	Astigmatism Mean ± SD D	Astigmatism Median (IQR) D	Astigmatism Range D	%Female
European adult cohorts							
1958 British Birth Cohort	White European	1645 (182/1463)	42 ± 0	0.47 ± 0.53	0.38 (0.13 – 0.63)	0.00 – 4.50	45.8%
ALSPAC Mothers	White European	1889 (343/1546)	44 ± 2	0.63 ± 0.53	0.50 (0.25 – 0.75)	0.00 – 4.62	100.0%
AREDS	White European	1864 (567/1297)	68 _ 5	0.77 ± 0.67	0.75 (0.25 – 1.00)	0.00 – 4.50	59.2%
BATSplusTEST	White European	204 (49/155)	40 ± 14	0.63 ± 0.57	0.38 (0.25 – 0.89)	0.00 – 2.75	62.7%
CROATIA-Korcula	White European	826 (135/691)	56 ± 13	0.63 ± 0.52	0.50 (0.25 – 0.75)	0.00 – 4.00	64.7%
CROATIA-Split	White European	343 (35/308)	51 ± 13	0.55 ± 0.41	0.44 (0.25 – 0.63)	0.00 – 3.00	56.3%
CROATIA-Vis	White European	529 (104/425)	56 ± 13	0.68 ± 0.57	0.51 (0.21 – 0.81)	0.00 – 4.68	59.7%
ERF4	White European	2485 (472/2013)	49 ± 14	0.58 ± 0.54	0.50 (0.25 – 0.75)	0.00 – 4.13	43.4%
FITSA	White European	87 (18/69)	68 ± 3	0.75 ± 0.52	0.63 (0.38 – 0.88)	0.00 – 3.50	100.0%
Framingham	White European	1532 (745/787)	60 ± 12	0.78 ± 0.56	0.63 (0.38 – 1.00)	0.00 – 4.38	56.1%
GUTENBERG	White European	3954 (640/3314)	56 ± 11	0.55 ± 0.54	0.44 (0.13 – 0.75)	0.00 – 4.63	49.2%
KORA	White European	1852 (448/1404)	56 ± 12	0.72 ± 0.64	0.50 (0.25 – 1.00)	0.00 – 4.75	50.6%
OGLIASTRA	White European	472 (49/423)	52 ± 16	0.31 ± 0.52	0.00 (0.00 – 0.50)	0.00 – 3.00	69.0%
ORCADES	White European	502 (113/389)	58 ± 14	0.70 ± 0.65	0.56 (0.22 – 0.90)	0.00 – 4.69	56.8%
ROTTERDAM 1	White European	5422 (2193/3229)	69 ± 9	0.95 ± 0.66	0.75 (0.38 – 1.13)	0.00 – 4.75	58.6%
ROTTERDAM 2	White European	1973 (725/1248)	64 ± 7	0.89 ± 0.59	0.75 (0.44 – 1.07)	0.00 – 4.50	54.3%
ROTTERDAM 3	White European	1971 (580/1391)	56 ± 6	0.81 ± 0.57	0.63 (0.31 – 0.94)	0.00 – 4.00	56.5%
TwinsUK	White European	2658 (751/1907)	55 ± 13	0.80 ± 0.65	0.63 (0.38 – 1.00)	0.00 – 4.88	91.1%
WESDR adults	White European	280 (69/211)	35 ± 8	0.71 ± 0.65	0.50 (0.19 – 0.81)	0.00 – 4.50	75.4%
YFS	White European	1480 (269/1211)	42 ± 5	0.64 ± 0.52	0.50 (0.25 – 0.75)	0.00 – 4.13	55.3%
Asian adult cohorts							
BES	Chinese	585 (154/431)	62 ± 9	0.66 ± 0.59	0.50 (0.25 – 1.00)	0.00 – 3.50	65.8%
HK-MGS adults	Chinese	120 (59/61)	34 ± 7	1.29 ± 1.05	0.97 (0.50 – 1.84)	0.00 – 5.31	61.7%
SCES	Chinese	1662 (670/992)	57 ± 9	0.99 ± 0.63	0.85 (0.48 – 1.23)	0.00 – 4.30	48.8%
SIMES	Malay	2165 (706/1459)	57 ± 11	0.90 ± 0.66	0.73 (0.39 – 1.06)	0.00 – 4.85	50.8%
SINDI	Indian	1998 (739/1259)	56 ± 9	0.96 ± 0.62	0.83 (0.47 – 1.18)	0.00 – 4.53	48.7%
SP2	Chinese	1954 (543/1411)	48 ± 11	0.81 ± 0.56	0.68 (0.36 – 0.0.99)	0.00 – 4.18	54.2%
STARS	Chinese	811 (205/606)	39 ± 5	0.72 ± 0.67	0.60 (0.21 – 0.94)	0.00 – 4.32	48.0%
European youngsters cohorts							
ALSPAC children	White European	3828 (580/3248)	15 ± 0.3	0.65 ± 0.42	0.63 (0.38 – 0.75)	0.00 – 4.25	48.8%
BATSplusTEST children	White European	561 (60/501)	18 ± 4	0.40 ± 0.48	0.25 (0.13 – 0.5)	0.00 – 4	54.0%
RAINE	White European	1007 (215/792)	20 ± 0	0.74± 0.40	0.69 (0.45-0.93)	0.08 – 3.11	49.3%
WESDR children	White European	244 (52/192)	18 ± 4	0.64 ± 0.57	0.50 (0.25 – 0.75)	0.00 – 3.38)	50.8%
Asian youngsters cohort							
SCORM	Chinese	917 (247/670)	11 ± 1	0.77 ± 0.66	0.57 (0.21 – 0.94)	0.00 – 4.32	48.0%

Phase 2) with either MACH[64] or IMPUTE[65]. SNPs that passed cohort-specific QC metrics were used as a framework for imputation, and reference haplotypes were chosen from the best available HapMap Phase 2 ancestry group[28]. See Supplemental Methods and Table S1b for more details.

Statistical analysis

A GWAS was carried out separately for each participant cohort. SNPs were tested individually for association with astigmatism in a logistic regression model, with case/control status as the dependent variable. SNP imputed dosage was modelled as a linear covariate (on a continuous scale from 0 – 2) where one allele was assigned as the reference allele and the other allele the risk allele. Age and sex were included as additional covariates when appropriate. If significant population stratification was detected in a cohort, then either the first two principal components (PCs) were included in the logistic regression or an analysis method was used that jointly adjusted for population stratification and cryptic relatedness as part of the analysis. This approach is commonly used in GWAS meta-analysis[66-68]. Details of the GWAS analyses performed in each cohort are given in Supplemental Methods. SNPs were carried forward for meta-analysis if they met the following criteria of a MAF >1%, and an OR (odds ratio) between 0.2 and 5.0 (the latter step being included to remove SNPs with an OR of approximately zero or infinity, which occurred for a few SNPs in the smaller cohorts due to low minor allele counts). Effect estimates were reported with reference to the positive strand of the NCBI Build 36 reference sequence of the human genome. Meta-analysis was carried out using a fixed-effects model with METAL [69]. For the meta-analysis of all cohorts, the adult ALSPAC sample was excluded because, given the inclusion of the ALSPAC young persons sample (biological relatives of the adults), this could have led to falsely-inflated estimates of association. The number of subjects contributing information to the meta-analysis summary statistic varied, as shown in Tables 2 and 3. This occurred primarily through markers being monomorphic (uninformative) in certain samples, and to a small extent through missing data for certain markers in specific individuals. A P-value <5.0E-08 was used to declare genome-wide significance[70,71].

RESULTS

Meta-analyses of refractive astigmatism GWAS results were carried out for 3 subject groups: White Europeans aged ≥25 years, White European subjects aged <25 years, and Asians aged ≥25 years. There was little evidence of population stratification in any of the meta-analysis results datasets (Genomic Control lambda, λ_{GC}=1.014, 1.011, 1.018 and 1.022 for White Europeans aged ≥25 years, White European subjects aged <25 years, Asians aged ≥25 years, and all samples combined, respectively).

Meta-analysis of White Europeans aged at least 25 years

For the meta-analysis of older White European individuals (n= 31,968) there were 6 regions containing markers with *P*-values <5.0E-06, suggestive of association (Table 2; Figures. 1 & 2). However, only a single region contained markers that met the *P*-value conventionally accepted to declare genome-wide significance (P <5.0E-08). This was at 2p16.3, downstream of the gene encoding neurexin-1 (*NRXN1*; Figure 2A) with the most strongly associated marker being

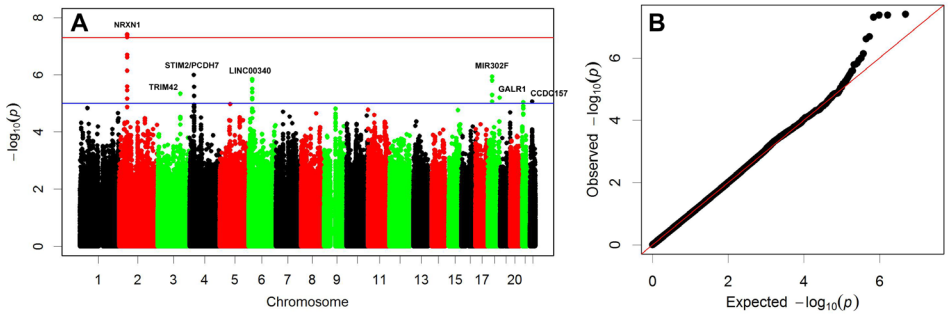

Figure 1. Results of the meta-analysis of White European subjects aged ≥ 25 years old.
Panel A: Manhattan plot of log *P*-values against genomic position. The red horizontal line is the threshold commonly used to for declaring genome-wide significance (*P*=5.0E-08). The blue line indicates *P*=1.0E-05. Genes adjacent to the association signal are indicated. Panel B: Quantile-quantile (QQ) plot of observed versus expected distribution of log *P*-values. The red line shows the distribution expected by chance.

rs1401327. Each copy of the A allele of rs1401327 increased the odds of astigmatism with an OR= 1.16 (95% CI: 1.10 to 1.22; *P*=3.92E-08). The next most strongly associated regions were at 3q23, 4p15, 6p22.3, and 18q12.1 (Table 2). There was little evidence of heterogeneity of effect across cohorts at any of the above loci (I^2 <14; Table 2).

Meta-analysis of White Europeans aged less than 25 years

The meta-analysis of younger White European cohorts identified 4 regions with P-values below 5.0E-06 (Table 2). However, the much smaller sample size (n=5,640) meant that this meta-analysis had limited statistical power to detect true-positive associations. The most strongly associated SNP was rs1366200 (OR=1.31, 95% CI=1.17 to 1.46; P=1.04E-06) within the *AQPEP* gene on chromosome 5q23.1.

Meta-analysis of Asians aged at least 25 years

In the meta-analysis of Asian cohorts (n=9,295) the most strongly associated marker was rs7534824 (OR=2.30, 95%CI=1.65 to 3.22; P=9.00E-07) within a gene of unknown function, *LOC101928334*, on chromosome 1. This marker had a low allele frequency (MAF=0.03). Two other regions also contained SNPs with P-values <5.0E-06 (Table 2). However, this meta-analysis also had limited statistical power to detect true-positive associations.

Meta-analysis of all cohorts

In order to search for evidence to corroborate the initial findings, we carried out a meta-analysis of all independent individuals from the above 3 cohort groups combined with Asians <25 years of age from the SCORM study (n= 45,931). As shown in Table 3, this revealed little evidence across cohort groups to substantiate the initial findings. The three most strongly associated regions were the *NRXN1* locus, the *TOX* gene locus on chromosome 8q12.1, and the *LINC00340* gene locus at 6p22.3, all of which were amongst the most highly-associated regions identified in the meta-analysis of older White European subjects. Association at the *NRXN1* gene locus (rs1401327,

Table 2. Most strongly associated SNPs in the 3 meta-analyses. The table shows all SNPs with P < 5.0E-06.

	SNP	Chr	Pos	RA	NRA	RAF (min – max)	OR	95%CI	P-value	I^2	n	**Gene(s)**
European subjects aged ≥25 years	rs1401327	2	49900987	A	G	0.113-0.174	1.157	1.098-1.218	3.92E-08	0	31694	*NRXN1*
	rs17795388	2	49900356	G	A	0.113-0.174	1.157	1.098-1.218	4.16E-08	0	31691	*NRXN1*
	rs11690625	2	49908115	C	A	0.113-0.175	1.156	1.098-1.218	4.17E-08	0	31731	*NRXN1*
	rs17795358	2	49897928	A	G	0.113-0.173	1.156	1.097-1.218	4.94E-08	0	31672	*NRXN1*
	rs925931	2	49913312	C	T	0.113-0.173	1.148	1.090-1.210	2.06E-07	0	31727	*NRXN1*
	rs885560	2	49909442	G	A	0.113-0.175	1.146	1.088-1.207	2.46E-07	0	31728	*NRXN1*
	rs6708111	2	49878453	A	C	0.102-0.168	1.139	1.082-1.200	7.27E-07	0	31531	*NRXN1*
	rs11690252	2	49890187	T	G	0.230-0.342	1.105	1.060-1.151	2.59E-06	0	31511	*NRXN1*
	rs1878856	2	49877706	C	T	0.214-0.336	1.105	1.059-1.153	3.56E-06	0	31603	*NRXN1*
	rs12638075	3	1.42E+08	C	T	0.014-0.024	1.376	1.200-1.577	4.69E-06	0	27304	*TRIM42/CLSTN2*
	rs2309717	4	27859336	A	C	0.089-0.170	1.143	1.083-1.206	1.02E-06	11.8	31143	*STIM2/PCDH7*
	rs2871434	4	29931147	T	A	0.095-0.154	1.14	1.079-1.204	2.66E-06	13.6	31664	*STIM2/PCDH7*
	rs12212674	6	22195053	A	T	0.496-0.621	1.099	1.058-1.142	1.45E-06	0	31691	*LINC00340*
	rs6901423	6	22194271	G	A	0.496-0.621	1.099	1.057-1.142	1.63E-06	0	31689	*LINC00340*
	rs4712652	6	22186594	A	G	0.495-0.687	1.097	1.055-1.141	3.13E-06	0	28910	*LINC00340*
	rs9366427	6	22204592	G	C	0.487-0.619	1.094	1.053-1.136	4.15E-06	0	31773	*LINC00340*
	rs4799964	18	26239477	G	T	0.020-0.048	1.267	1.152-1.394	1.16E-06	0	31881	*MIR302F*
	rs12607243	18	26229228	G	A	0.020-0.050	1.264	1.149-1.392	1.60E-06	0	31882	*MIR302F*

European subjects aged <25 years	rs6688613	1	165218493	T	C	0.240-0.253	1.309	1.170-1.465	2.68E-06	0	5640	*MAEL*
	rs1327866	1	165219534	G	A	0.238-0.253	1.308	1.169-1.464	2.89E-06	0	5640	*MAEL*
	rs7550698	1	165217705	C	T	0.240-0.253	1.308	1.168-1.463	3.02E-06	0	5640	*MAEL*
	rs7528849	1	165221494	G	A	0.240-0.253	1.307	1.168-1.463	3.11E-06	0	5640	MAEL
	rs7518155	1	165221520	G	T	0.240-0.253	1.307	1.168-1.462	3.19E-06	0	5640	*MAEL*
	rs7545911	1	165214305	A	G	0.240-0.253	1.309	1.169-1.467	3.35E-06	0	5640	*MAEL*
	rs6682062	1	165216603	C	G	0.240-0.253	1.309	1.168-1.467	3.39E-06	0	5640	*MAEL*
	rs2296837	1	165225225	C	T	0.240-0.253	1.305	1.166-1.461	3.53E-06	0	5640	*MAEL*
	rs11578336	1	165225334	G	T	0.240-0.253	1.304	1.166-1.460	3.71E-06	0	5640	*MAEL*
	rs1366200	5	115349718	G	T	0.312-0.321	1.308	1.174-1.457	1.04E-06	48.7	5640	*AQPEP*
	rs17712049	7	48236741	C	T	0.875-0.904	1.569	1.295-1.902	4.39E-06	0	5640	*ABCA13*
	rs13257518	8	32755116	A	T	0.177-0.217	1.37	1.202-1.561	2.36E-06	15.7	5640	*NRG1*
	rs10503929	8	32733525	C	T	0.167-0.215	1.352	1.192-1.534	2.68E-06	32.3	5640	*NRG1*
	rs2975500	8	32724907	A	G	0.110-0.161	1.435	1.231-1.673	3.95E-06	0	5640	*NRG1*
Asian adults	rs7534824	1	101394034	A	G	0.967-0.974	2.304	1.651-3.214	9.00E-07	0	4812	*LOC101928334*
	rs10496034	2	54998439	C	G	0.170-0.287	1.216	1.122-1.318	2.13E-06	0	8780	*EML6*
	rs428445	20	54469954	T	G	0.713-0.954	1.314	1.175-1.470	1.84E-06	0	8908	*CASS4/GCNT7*
	rs6999	20	54527308	A	G	0.713-0.957	1.303	1.164-1.459	4.30E-06	0	8904	CASS4/GCNT7

RA=risk allele; NRA=non-risk (reference) allele; RAF=risk allele frequency in each cohort; OR=odds ratio; 95% CI=95% confidence interval of odds ratio; I^2=heterogeneity statistic; n=total sample size.

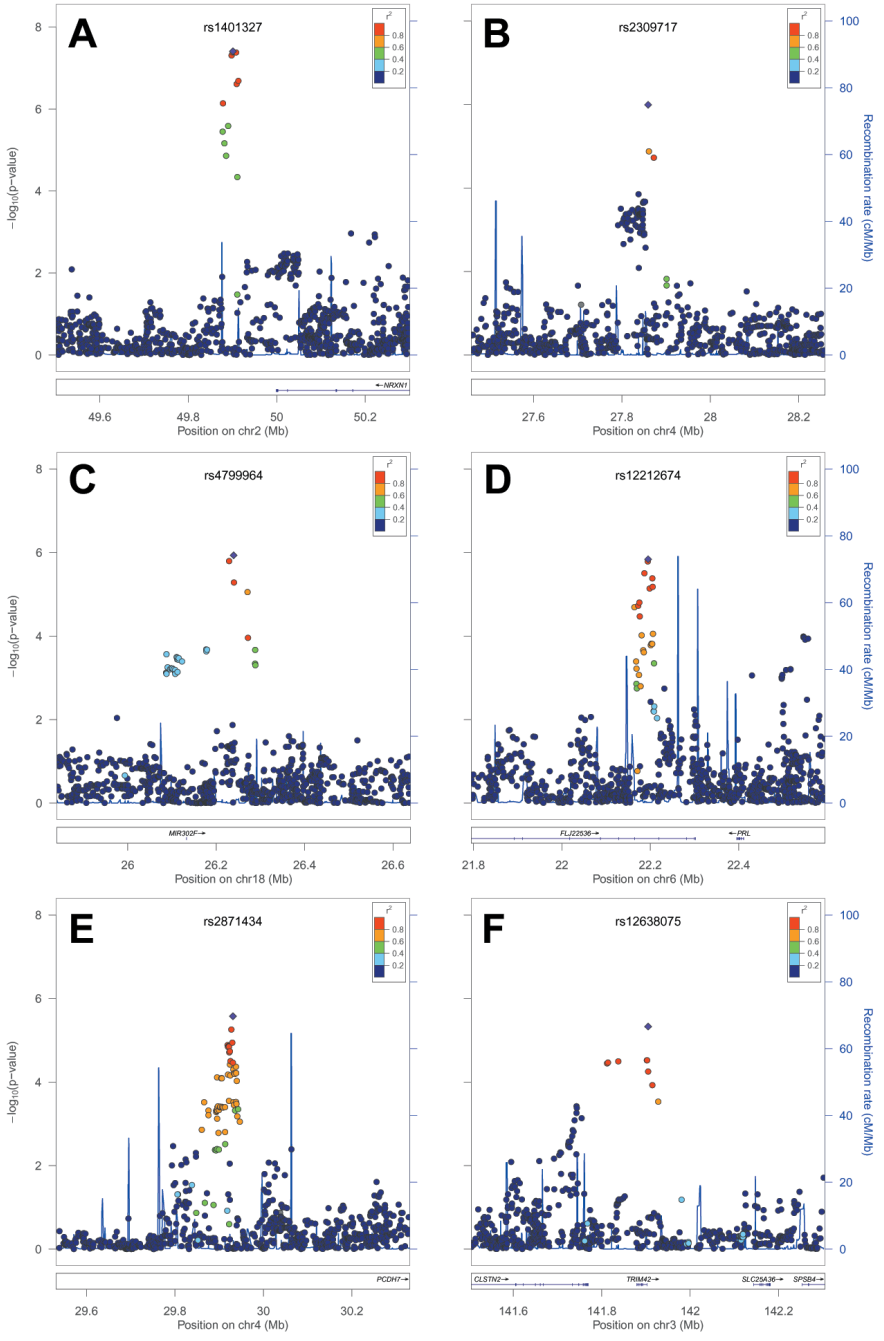

Figure 2. Regions showing the strongest evidence for association with refractive astigmatism in the meta-analysis of White Europeans aged ≥ 25 years

Table 3. Most strongly associated SNPs in the meta-analysis of all cohorts. The table shows all SNPs with P < 5.0E-06.

SNP	Chr	Pos	RA	NRA	RAF (min – max)	OR	95%CI	P-value	I^2	n	**Gene**
rs1401327	2	49900987	A	G	0.113-0.174	1.139	1.084-1.198	2.93E-07	0	35445	*NRXN1*
rs11690625	2	49908115	C	A	0.113-0.175	1.139	1.084-1.197	2.95E-07	0	35482	*NRXN1*
rs17795388	2	49900356	G	A	0.113-0.174	1.139	1.084-1.198	3.10E-07	0	35442	*NRXN1*
rs17795358	2	49897928	A	G	0.113-0.173	1.139	1.083-1.197	3.67E-07	0	35423	*NRXN1*
rs925931	2	49913312	C	T	0.010-0.173	1.125	1.071-1.182	2.64E-06	3.3	39567	*NRXN1*
rs885560	2	49909442	G	A	0.010-0.175	1.123	1.069-1.179	3.46E-06	5.5	39566	*NRXN1*
rs6708111	2	49878453	A	C	0.102-0.168	1.124	1.069-1.182	4.42E-06	0	35282	*NRXN1*
rs7581641	2	8543557	T	C	0.012-0.103	1.225	1.123-1.336	4.74E-06	0	41865	*NRXN1*
rs6892230	5	65175520	A	G	0.016-0.078	1.236	1.133-1.349	1.87E-06	41.3	37591	*NLN*
rs12212674	6	22195053	A	T	0.134-0.621	1.086	1.050-1.123	1.49E-06	0	45134	*LINC00340*
rs6901423	6	22194271	G	A	0.134-0.621	1.083	1.048-1.120	3.00E-06	0	45132	*LINC00340*
rs1034071	6	22205354	C	T	0.137-0.608	1.081	1.046-1.118	3.73E-06	0	45330	*LINC00340*
rs7823467	8	60241288	T	C	0.388-0.713	1.085	1.052-1.120	3.47E-07	22.9	45273	*TOX*
rs10086929	8	60252851	A	G	0.430-0.709	1.083	1.049-1.118	7.36E-07	22.3	45156	*TOX*
rs6471768	8	60230697	T	A	0.435-0.710	1.082	1.048-1.117	1.07E-06	23.9	45125	*TOX*
rs4531042	8	60251242	G	A	0.388-0.737	1.082	1.048-1.118	1.45E-06	32.9	45277	*TOX*
rs4738757	8	60218783	A	G	0.388-0.701	1.08	1.046-1.115	1.89E-06	26.9	45122	*TOX*
rs12675886	8	60309643	C	T	0.458-0.704	1.079	1.045-1.114	2.50E-06	14.3	45082	*TOX*
rs6997378	8	60330443	T	G	0.460-0.705	1.077	1.043-1.111	4.95E-06	17.3	45085	*TOX*
rs1944146	11	130195372	A	G	0.524-0.608	1.08	1.046-1.115	2.62E-06	17.3	45243	*LOC100507431*
rs7934985	11	130194532	G	A	0.523-0.613	1.08	1.046-1.116	2.66E-06	4.8	45123	*LOC100507431*

OR=1.139, 95% CI: 1.084-1.198, *P*=2.93E-07) was driven solely by the European cohorts, since the associated SNPs were monomorphic in Asians, and thus uninformative. The most strongly-associated marker at the *TOX* gene locus was rs7823467 (OR=1.09, 95% CI: 1.05-1.12; *P*=3.47E-07) while that at the *LINC00340* gene locus was rs12212674 (OR=1.09, 95% CI: 1.05-1.12; *P*=1.49E-06).

Interestingly, the *TOX* region is one of the loci identified in the CREAM consortium GWAS for spherical equivalent refractive error[28] and the age-of-onset of myopia GWAS carried out by 23andMe[29]. Therefore to investigate whether spherical refraction and astigmatism share common genetic determinants more widely, we examined the association with refractive astigmatism of 34 genome-wide significant SNPs (Table S1) reported in the CREAM[28] and 23andMe[29] spherical equivalent GWAS meta-analyses (4 additional SNPs associated with spherical equivalent could not be included since they were not analysed in the current study). For each SNP, the effect size (beta coefficient describing the magnitude of association) with spherical equivalent was plotted against the effect size for association with refractive astigmatism (Figure 3). The betas were found

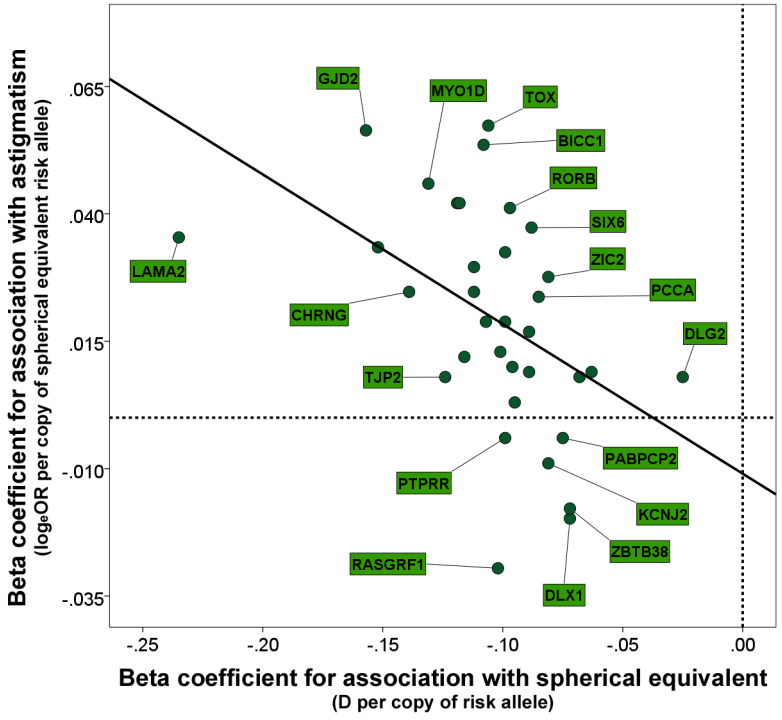

Figure 3. Common genetic determinants for spherical equivalent refractive error are shared with refractive astigmatism.
GWAS meta-analysis beta coefficients (which quantify the effect size of SNPs) were compared between studies of spherical equivalent and refractive astigmatism. The SNP beta coefficients for spherical equivalent were obtained from the CREAM consortium GWAS for spherical equivalent[28], while those for refractive astigmatism were from the current study. The 34 SNPs analysed were chosen based on prior genome-wide significant evidence of association with spherical equivalent in the CREAM[28] and 23andMe[29] GWAS meta analyses. The solid line is the best linear fit to the data.

to be highly correlated (r=-0.59, *P*=2.10E-04). Excluding the SNP in the *TOX* gene region had minimal influence on the correlation of the betas for the remaining 33 SNPs (r=-0.60, *P*=2.29E-04).

DISCUSSION

This GWAS meta-analysis of nearly 46,000 individuals identified several novel, suggestive candidate genes/regions for refractive astigmatism, including *NRXN1*, *TOX* and *LINC00340*. One of these regions, near the *NRXN1* gene region, reached genome-wide significance in the White European adult group. Two thirds of the ~46,000 subjects included in the full meta-analysis were White European adults and so the results are likely to have been driven mainly by this group. Therefore, until the opportunity arises for replication in independent samples, especially in large numbers of comparable White European adults, caution is needed in interpreting these results. These results should not be considered to be relevant to other populations until replicated in younger White European samples or in other ethnic groups.

Novel candidate genes underlying the observed associations

Neurexin-1, one of the largest genes in the human genome, is thought to function in cell adhesion, as well as synapse development and maintenance[72,73]. Structural genomic deletions that delete or disrupt *NXRN1* are strongly implicated in causing psychiatric and cognitive phenotypes including schizophrenia, autism and mental retardation[74]. To our knowledge these conditions are not known to be associated with refractive astigmatism although refractive errors, in general, are more prevalent in individuals with learning difficulties[75]. A recent survey of 25 patients with exonic deletions involving the gene for neurexin-1[74] unfortunately did not describe these patients' ocular features. While the strength of association reached genome-wide significance in the adult European sample (n=31,968, *P*=3.92E-08), this weakened when the younger European subjects were included (n=35,719, *P*=2.93E-07) while having little impact on the estimated effect size (OR=1.16 and OR=1.14, respectively). The associated SNPs in this region were monomorphic in Asian subjects, suggesting they arose relatively recently in human evolution.

The associated variants at 8q12.1 lie upstream of the *TOX* promoter and thus would be well-placed to influence its transcription level. However it is not clear whether *TOX* or a nearby gene mediates this locus' impact on spherical equivalent refractive error – and potentially astigmatism. The known roles of *TOX* relate to immune function, which argues against a role in refractive development and instead suggests that another gene such as *SDCBP* (syndecan binding protein) also known as syntenin, which lies 600 kb from the most-strongly associated marker may be involved. Syntenin acts as a link between the proteoglycan/matrix receptor syndecan-1 and the cytoskeleton, and its proposed functions include cell adhesion. Furthermore, syntenin-null mice show wound healing defects that are particularly marked in the cornea[76,77].

The 6p22.3 locus containing the long intergenic non-coding RNA gene *LINC00340* (also known as *FLJ22536* and *CASC15*) is gene-poor (Figure 2D) yet has previously shown association with aggressive neuroblastoma in GWAS studies[78]. The mechanisms through which non-coding RNAs act are poorly understood[79,80] but in the case of lincRNAs the mechanism may involve epigenetic regulation[81]. No obvious candidate astigmatism susceptibility gene is present in this genomic

location. As with *NRXN1*, the association with *LINC00340* was almost wholly driven by the adult European cohorts (*P*=1.45E-06 versus *P*=1.49E-06 in all cohorts combined).

As well as *NRXN1* and *SDCBP*, additional genes in the most strongly associated regions have putative roles in cell adhesion and/or synapse function. The gene nearest to the lead SNP at 3q23 in European adults (rs12638075, *P*=4.69E-06) is *TRIM42* (tripartite motif containing-42). Because members of the *TRIM* gene family function mostly in immune signalling[82], the adjacent gene *CLSTN2* (calsyntenin-2; also known as cadherin-related family member-13) is potentially of greater interest given its proposed role in cell adhesion and synapse function[83]. Furthermore, the association described above for markers in the vicinity of the *SDCBP* gene, encoding syntenin, lends support to the putative involvement of *CLSTN2*. One of the two regions on chromosome 4p15 (lead SNP rs2871434; Figure. 2E) contains the *PCDH7* (protocadherin-7) gene, which given its role in cell adhesion is a plausible candidate gene for astigmatism. In mice homozygous for a null allele of the *EGR1* gene, which develop a transient axial myopia postnatally, a member of the protocadherin gene family, *Pcdhb9*, was the most highly differentially expressed retinal gene when compared to wild type mice[84]. The second associated region at 4p15 (lead SNP rs2309717; Figure. 2B) contains no known genes - the closest being *MIR4275*, which lies 600 kb away. However, amongst the more than 6000 predicted targets of miR-4275 is the nearby *PCDH7*.

Genetic co-determination of spherical equivalent and refractive astigmatism

One of the most exciting findings from this study was the evidence for overlap in genetic susceptibility between spherical and astigmatic refractive errors (Figure 3). It is well-known that spherical and astigmatic refractive errors tend to co-occur[8,85]. However, to our knowledge this is the first study to provide evidence supporting shared genetic susceptibility for the two traits. Kee and co-workers[6,86] have shown in monkeys and chickens that visual experience can alter spherical equivalent and astigmatic refractive errors concurrently. Hence, in line with the view that genetic factors might alter refractive development by regulating how the eye responds to visual cues[87,88], it is feasible that causal variants tagged by the SNPs examined here impact on both spherical equivalent and astigmatism via visual feedback.

The suggestive findings here that genes related to cell adhesion and synapse function may be involved in susceptibility to astigmatism is also consistent with the concept of genetic co-determination of spherical equivalent and refractive astigmatism, as several candidate genes identified in GWAS for spherical equivalent refractive error have putative roles in synapse function or plasticity, for example *RASGRF1*, *GRIA4*, *RBFOX1*, *LRRC4C*, *DLG2*[27-29,89] as well as in cell adhesion, for example *TJP2*, *CTNND2*, *ANTXR2*, and *LRFN5*[28,29,90].

Comparison with previous work and limitations of the current study

Results from the meta-analysis of all cohorts for SNPs previously associated with astigmatism are shown in Table 4. Because the cohorts studied here overlap substantially with those examined previously[15,16], low *P*-values were expected – but not found. Thus the *P*-values in Table 4 provide little evidence for replication of the previously associated markers. This is especially surprising for the corneal astigmatism-associated SNP at the *PDGFRA* locus[15], since this has already

Table 4. Results from the meta-analysis of all cohorts for SNPs previously associated with corneal astigmatism (CA) or refractive astigmatism (RA).

Trait	SNP	Chr	RA	NRA	RAF (min – max)	OR	95%CI	P-value	I^2	N	Gene	**Reference**
RA	rs3771395	2	A	G	0.06-0.30	1.04	1.00-1.09	5.17E-02	19.2	45,324	*VAX2*	Lopes et al.[16]
CA	rs7677751	4	T	C	0.07-0.26	1.03	0.99-1.08	1.03E-01	17.9	45,287	*PDGFRA*	Fan et al.[15]
RA	rs795544	5	C	A	0.64-0.92	1.05	1.01-1.09	2.01E-02	0	45,245	*DNAH5*	Lopes et al.[16]
RA	rs10226930*										*SHH*	Lopes et al.[16]
RA	rs485842	11	C	T	0.33-0.77	1.05	1.01-1.08	1.21E-02	10.2	45,137	*MAML2*	Lopes et al.[16]
RA	rs12445126	16	A	G	0.02-0.14	1.02	0.97-1.09	4.16E-01	21.1	45,198	*XYLT1*	Lopes et al.[16]
RA	rs11644988	16	G	A	0.73-0.99	1.04	0.98-1.11	2.46E-01	0	40,369	*FOXF1*	Lopes et al.[16]

*SNP not present in current meta-analysis.

been replicated in a cohort of differing ethnicity[24]. Instead, the lack of replication may reflect the different traits examined (*corneal* versus *refractive* astigmatism). The other SNPs previously associated with astigmatism did not reach genome-wide significance in the original study, and were associated with astigmatism when analyzed as a quantitative trait, which may explain the lack of independent replication.

Genetic studies of astigmatism are hampered by the variation in its magnitude and orientation with age, and its non-Gaussian frequency distribution, all of which complicate the choice of analysis model. In younger individuals, astigmatism is typically "with the rule" (WTR; axis of minus power cylindrical correcting lens close to horizontal) while in later life it usually switches to "against the rule" (ATR; correcting negative cylinder axis close to vertical)[5,85]. Amongst the theories explaining this transition, a loosening of eyelid tension is the most widely supported[8]. If it is the case that ATR and WTR astigmatism have different etiologies, then GWAS investigations should attain maximum statistical power by modelling younger and older subjects separately, modelling ATR and WTR astigmatism separately, or in modelling astigmatism as a vector quantity. However, the age-dependent shift in WTR to ATR largely concerns low-level astigmatism whereas higher levels may be more stable over the life course[91,92]. Thus, the present study adopted a dichotomous case/control classification scheme, and analyzed younger and older subjects separately, in an attempt to mitigate the effects of axis changes with age. The dichotomization scheme also allayed concerns regarding the non-normality of the trait, although this would have been at the expense of statistical power.

The use of a dichotomous phenotype definition for our GWAS meta-analysis of astigmatism contrasts with the quantitative trait approach used in previous GWAS meta-analyses by the CREAM consortium for refractive error and axial eye length[28,93]. It has been shown that binary trait GWAS meta-analysis results are sensitive to unequal numbers of cases and controls in individual cohorts, especially when the sample size is small[69]. However, we found very similar results when overcoming this potential source of bias by using an "effective sample size" rather than actual sample size during meta-analysis[69]. In addition to the problem of unequal case/control sample sizes, we also observed highly inflated type-I errors during initial meta-analysis trials due to extreme OR estimates for a small number of low MAF markers in certain cohorts, e.g. if the minor allele was present in controls but absent in cases. To circumvent this, we pre-screened each GWAS results file, excluding markers with unfeasibly high OR estimates (OR < 0.2 or OR >5.0).

Out of 7 Asian adult cohorts (total n=9,295), 5 were Chinese cohorts (n=5,132, about 55% of the total Asian adult sample). Therefore, we cannot generalize our results from the Asian adult group with ease. Importantly, the SNP (rs7534824, in the gene *LOC101928334*) which showed the strongest suggestive association in the Asian group was only polymorphic in the Chinese cohorts (monomorphic in the Indian and Malay cohorts). For the other 3 SNPs reported in Table 2, although they are polymorphic in all three ethnic groups, the association signal was mainly driven by the observed association in the 5 Chinese cohorts.

In summary, this large-scale meta-analysis of GWAS studies for refractive astigmatism identified only a single locus that reached genome-wide significance (2p16.3, near *NRXN1*, in European

adults) and there was no evidence for replication of this region in younger European individuals or in non-Europeans. Several putative candidate genes with functions relating to cell adhesion and/or synapse function were present in the next most strongly associated regions. Consistent with earlier work, all of the most strongly associated genetic variants identified had small effects, supporting the polygenic nature of genetic susceptibility to refractive astigmatism in the general population. Fewer candidate risk variants were discovered for refractive astigmatism than were found previously by the CREAM consortium for spherical equivalent refractive error[28], despite studying similar subject cohorts. Nevertheless, there was compelling evidence for shared genetic susceptibility for spherical and astigmatic refractive errors, implying that the co-occurrence of these traits is, at least in part, genetically determined.

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4.1

Increasing prevalence of myopia in Europe and the impact of education

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ABSTRACT

Objective

To investigate whether myopia is becoming more common across Europe and explore if rising education levels, an important environmental risk factor for myopia, might explain any temporal trend.

Design

Meta-analysis of population-based, cross-sectional studies from the European Eye Epidemiology Consortium (E^3).

Participants

The E^3 Consortium is a collaborative network of epidemiological studies of common eye diseases in adults across Europe. Refractive data were available on 61,946 participants from fifteen population-based studies performed between 1990-2013 with a median age range of 44-78 years.

Methods

Non-cycloplegic refraction, year of birth and highest educational level achieved were obtained for all participants. Myopia was defined as mean spherical equivalent ≤-0.75 diopters. A random effects meta-analysis of age-specific myopia prevalence was performed, with sequential analyses stratified by year of birth and highest level of educational attainment.

Main outcome measure

Variation in age-specific myopia prevalence for differing years of birth and educational level.

Results

There was a significant cohort effect for rising myopia prevalence across more recent birth decades; age-standardized myopia prevalence increased from 17.8% (95% Confidence Interval (CI) 17.6-18.1) to 23.5% (95% CI 23.2-23.7) in those born between 1910-39 compared to 1940-79 (p=0.03). Education was significantly associated with myopia; for those completing primary, secondary and higher education the age-standardized prevalences were 25.4% (CI 25.0-25.8), 29.1% (CI 28.8-29.5) and 36.6% (CI 36.1-37.2) respectively. While more recent birth cohorts were more educated, this did not fully explain the cohort effect. Compared to the reference risk of participants born in the 1920s with only primary education, either higher education or being born in the 1960's doubled the myopia prevalence ratio (2.43 (CI 1.26-4.17) and 2.62 (CI 1.31-5.00) respectively), whilst individuals born in the 1960s completing higher education had almost four times the reference risk, prevalence ratio of 3.76 (CI 2.21-6.57).

Conclusions

Myopia is becoming more common in Europe; while education levels have risen and are associated with myopia, higher education appears to be an additive rather than explanatory factor. Rising levels of myopia carry significant clinical and economic implications, with more people at risk of the sight threatening complications associated with high myopia.

INTRODUCTION

Myopia (near-sightedness) occurs when a distant object's image is formed anterior to the retinal plane, most commonly as result of an increased axial length. This results in blurred distant vision and, unlike hyperopia, requires refractive correction at all ages and severity for clear focus. Myopia is already the most common eye condition worldwide but the prevalence is significantly increasing, especially in Southeast Asia[1-3]. In Europe, Australia and the United States the prevalence of myopia appears to be lower[4,5], however there is evidence of a rising prevalence in the United States and elsewhere[6-8], particularly in young adults[9]. This is of concern as myopia, even when appropriately corrected, is associated with an increased risk of sight threatening diseases such as myopic maculopathy, retinal detachment, glaucoma and cataract[10]. Myopic maculopathy is currently untreatable and already contributes to visual impairment in working age adults[11]. Rising myopia levels in Europe would carry implications for public health policy both in the provision of clinical services and economic sequelae from the resulting visual impairment in the working population.

Myopia is a highly heritable trait[12,13] and to date a number of genetic polymorphisms have been associated with refractive error, albeit explaining a small proportion of this heritability[14,15]. Environmental factors play a key role in myopia development and must explain the recent changes in prevalence[16]. Myopia has been associated with education, near work, urbanisation, pre-natal factors, socio-economic status, cognitive ability, season of birth, light and time spent outdoors[2,16-25]. One of the strongest and most replicated risk factors is educational attainment[16,26], and there is some evidence of interaction between genetic factors and education to influence the risk of myopia[27]. The increased levels of higher education over the 20th Century[28] might be a causative factor, or marker of a causative factor, for rising myopia prevalence.

The aims of this study are to identify whether myopia is becoming more common across Europe and to examine whether rising levels of education explain any temporal trend, using data from over 60,000 participants from the European Eye Epidemiology (E^3) Consortium.

MATERIAL AND METHODS

Study population

The European Eye Epidemiology (E^3) consortium is a collaborative initiative to share and meta-analyse epidemiological data on common eye diseases across Europe. Thirty-three studies are currently part of the consortium and a range of ophthalmic data is available on approximately 124,000 individuals, from population-based and case-control cohorts. All studies adhere to the tenets of the Declaration of Helsinki, and relevant local ethical committee approvals with specific study consent were obtained.

Refractive error measurements were included from 68,350 adults within the fifteen E^3 population-based studies who had data on refractive error. These included population-based cross-sectional or cohort studies, with two studies recruiting participants nationally and thirteen recruited from a local population. Further detail on each study is provided in Table 1. Exclusion criteria included subjects who had cataract or refractive surgery, retinal detachment, or other conditions, such as

Table 1. Description of the European Eye Epidemiology Consortium studies included in this meta-analysis.

Study	Data collection period	Study design	Total with refraction	Refraction method	Exclusions (cataract surgery)	Total included	Median age, years (range)	Gender, % female	Ethnicity, % European (% Unknown)	Higher education, %	Crude myopia prevalence, %
Northern Europe											
1958 British Birth Cohort, UK	2002-2003	Population-based birth cohort (N)	2502	Autorefraction	7 (0)	2495	44 (44-46)	51.7	98.0 (9.2)	29.9	48.7
EPIC-Norfolk, UK	2004-2011	Population-based cross-sectional study (L)	8508	Autorefraction	1110 (971)	7444	67 (48-92)	54.5	99.7 (0)	17.9	23.0
Tromsø Eye Study, Norway	2007-2008	Population-based cohort (L)	6565	Autorefraction	773 (700)	5792	61 (38-87)	55.9	NA (100)	32.5	19.4
TwinsUK, UK	1998-2010	National twin cohort (N)	6245	Autorefraction	161 (61)	6095	55 (16-85)	91.2	98.2 (23.9)	22.3	31.4
Southern Europe											
Thessaloniki Eye Study, Greece	1999-2005	Cross-sectional population-based study (L)	2259	Subjective	316 (303)	1952	69 (60-94)	44.7	100 (0)	Unknown	14.2
Western Europe											
ALIENOR, France	2006-2008	Population-based cohort (L)	951	Autorefraction	333 (318)	618	79 (73-93)	56.6	NA (100)	20.0	16.7
ERF, Netherlands	2002-2005	Family-based cross-sectional study (L)	2708	Subjective	46 (45)	2662	49 (14-87)	55.1	100 (0)	16.9	21.2
Gutenberg Health Study, Germany	2007-2012	Population-based cohort (L)	14679	Autorefraction	610 (610)	14069	54 (35-74)	49.4	NA (100)	37.6	31.9
KORA, Germany	2004-2005	Population-based cohort (L)	3078	Autorefraction	706 (177)	2372	55 (35-84)	50.4	100 (0)	14.7	36.1
Montrachet, France	2009-2013	Population-based cohort (L)	1143	Autorefraction	584 (562)	576	81 (76-92)	57.5	NA (100)	Unknown	19.1
Rotterdam Study I, Netherlands	1990-1993	Population-based cohort (L)	6748	Subjective	182 (172)	6566	68 (55-106)	59.3	98.5 (2.0)	11.6	16.4
Rotterdam Study II, Netherlands	2000-2002	Population-based cohort (L)	2689	Subjective	110 (110)	2579	62 (55-99)	54.8	87.8 (0.1)	22.3	21.9
Rotterdam Study III, Netherlands	2005-2008	Population-based cohort (L)	3624	Subjective	94 (74)	3530	56 (46-97)	56.3	NA (100)	31.4	32.5
POLA, France	1995-1997	Population-based cohort (L)	2464	Autorefraction	157 (128)	2315	70 (60-93)	55.8	NA (100)	7.3	16.2
Mixed											
EUREYE: Norway, UK, France, Italy, Greece & Estonia	2000-2002	Population based cross-sectional survey in seven cities (L)	4187	Autorefraction or focimetry with subjective refraction	1305 (517)	2882	72 (65-95)	56.7	NA (100)	30.0	15.6
Total Cohort	1990-2013		68,350		6404 (4748)	61,946	62	57.6	98.1	36.0	25.8

Myopia ≤ -0.75 diopters (D); NA, not applicable

keratoconus, which might influence refraction (n=6404). Data on age at refraction and birth year was available for 61,946 individuals, with information on education level in 60,125 subjects. Participants were mainly middle to late age, 98% European descent (where ethnicity was known), predominantly from Northern and Western Europe, and refractive examinations were performed from 1990 to 2013 (Table 1).

Study variables

Non-cycloplegic refractions were performed on all individuals using subjective refraction, autorefraction or a combination of focimetry with subjective refraction. Spherical equivalent (SE) was calculated using the standard formula (SE = sphere + (cylinder/2)). Myopia was defined as ≤ -0.75 diopters (D). Myopia prevalence by age was calculated, using five and ten year age bands from ≥15 years to ≥90 years. To study the impact of education on myopia, given the variation in educational systems across Europe, we established a simplified three-tier level of education across all cohorts. Primary education was defined as those leaving school before 16 years of age, secondary education in those leaving education up to the age of 19 years of age and higher education in those leaving education at or after the age of 20. Those under the age of 20 at the time of refraction (and therefore unable to have reached the highest education tier) were excluded from this analysis to avoid misclassification bias.

We investigated the evidence for a cohort effect for rising myopia prevalence by observing variations in myopia prevalence within defined age bands. These analyses are focused on the age range constituting the majority of our cohort (40-80 years of age, birth year 1910-1979, n=56,088), meaning the youngest and oldest participants, for whom we had no comparative birth cohort, were not considered. Prevalence was examined between different birth cohorts, initially using decade bins (1910 to 1970) and subsequently in two birth cohort groups divided by the median birth decade (1940-49). Finally we examined the influence of education by examining the myopia prevalence between birth cohorts with the additional stratification of educational status.

Statistical analysis

Study-specific summary data for myopia prevalence were obtained and combined in a random effect meta-analysis stratified by age. A random effects model was chosen over a fixed effects model, to allow for expected heterogeneity between studies as a result of varying study design. Age-standardization was performed with demographic distribution adjustments to age-specific estimates according to the European Standard Population 2010[29]. Evidence for the presence of a cohort effect was investigated using random effect meta-analyses of myopia prevalence stratified by age and birth year, and subsequently age, birth year and educational level. Differences between estimates of myopia prevalence were evaluated using the ANOVA test, proportion z tests and prevalence ratios (relative difference in prevalence against a defined baseline). Differences were considered significant at $p<0.05$.

Statistical analysis was performed using Stata version 13.1 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP). Graphical outputs were obtained using either Stata, Origin v9.0 (OriginLab Corporation 2013, Northampton, MA) or ggplot2[30] in R (R Core

Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (URL http://www.R-project.org)).

RESULTS

In this meta-analysis of 61,946 adults the overall myopia prevalence was 24.3% (95% Confidence Interval (CI) 20.1 - 28.5), with an age-standardised prevalence in Europe of 30.6% (95% CI 30.3-30.8). Age stratified analyses revealed a high prevalence in young adults (47.2% (95% CI 41.8-52.5) in those aged 25-29 years), which was almost double the prevalence in those of middle to older age (27.5% (95% CI 23.5-31.5) in those aged 55-59 years). There were no significant differences in the myopia prevalence by gender.

Cohort effect for rising myopia prevalence

There was a trend of higher myopia prevalence with more recent birth decade across all age groups (Figure 1), although sample sizes for some point estimates were low, resulting in wide confidence intervals (Table 2).

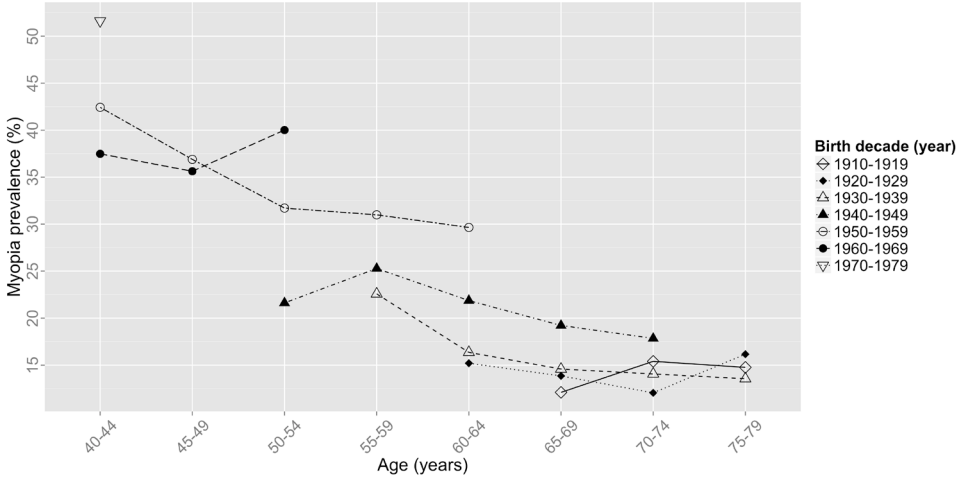

Figure 1. Prevalence of myopia (spherical equivalent ≤ -0.75 diopters) against age stratified by decade of birth. Individuals aged 40 to 79 included.

We examined the prevalence of myopia in two birth cohort groups (divided by the median birth decade): those born between 1910-1939 (n=22,660) and those born between 1940-1989 (n=33,428) (Figure 2). Myopia prevalence in a variance model was significantly higher in the more recent birth cohort group (p=0.03). Age-standardized myopia prevalence over a comparable age range of 50-79 years, increased from 17.8 % (95% CI 17.6-18.1) in those born 1910-1939 to 23.5% (95% CI 23.2-23.7) in those born 1940-1979. In age-specific analyses the prevalence of myopia in 50-59 year-olds was 22.5% (95% CI 20.2-24.9) in those born before 1940, compared to 29.2% (95% CI 25.3-33.0) in those born after 1940 (p=0.004). A similar significant rise of 15.3% (95% CI 13.4-17.3) to 21.2% (95% CI 18.6-23.8) was observed in those aged 60-69 years old (p<0.001).

Table 2. Prevalence of myopia (Spherical Equivalent ≤ -0.75 Diopters) against birth year stratified by age. Individuals aged 40 to 79 included.

	Birth decade						
Age	**1910-1919**	**1920-1929**	**1930-1939**	**1940-1949**	**1950-1959**	**1960-1969**	**1970-1979**
40-44					**42.4 (21.6-63.2)**	**37.5 (25.7-49.3)**	**51.6 (22.4-80.9)**
45-49				*	**36.9 (27.6-46.2)**	**35.6 (28.5-42.8)**	
50-54				**21.6 (14.2-29.1)**	**31.7 (27.0-36.5)**	**40.0 (33.0-47.0)**	
55-59			**22.6 (20.2-25.0)**	**25.3 (21.9-28.7)**	**31.0 (28.1-33.9)**		
60-64		**15.2 (12.7-17.7)**	**16.4 (13.2-19.5)**	**21.9 (18.8-25.0)**	**29.7 (18.9-40.4)**		
65-69	**12.1 (9.5-14.7)**	**13.9 (11.2-16.5)**	**14.6 (12.0-17.1)**	**19.2 (16.5-21.9)**			
70-74	**15.4 (13.0-17.8)**	**12.1 (8.8-15.3)**	**14.1 (11.4-16.7)**	**17.8 (10.9-24.8)**			
75-79	**14.8 (10.9-18.6)**	**16.2 (13.6-18.7)**	**13.6 (10.4-16.7)**				

(* = meta-analysis not possible due to single contributing prevalence estimate)

The influence of education on myopia risk & the cohort effect

The association between education and myopia was investigated in the thirteen studies for which these data were available (n=60,125 participants). Educational level was significantly associated with myopia prevalence across all age strata (p<0.0001). Overall the age-standardized myopia prevalence for those completing primary, secondary and higher education were 25.4% (95% CI 25.0-25.8), 29.1% (95% CI 28.8-29.5) and 36.6% (95% CI 36.1-37.2) respectively. There was an approximate two-fold increase in age-specific myopia prevalence between participants with primary compared to those with higher education in those aged 35-84 (the majority of study subjects) (Figure 3). For example, in subjects aged 45-49 when tested, the myopia prevalence

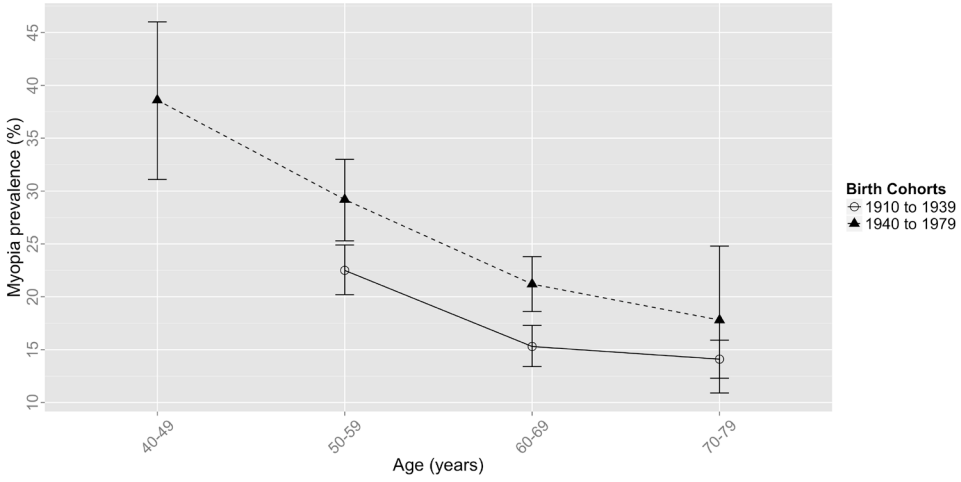

Figure 2. Prevalence of myopia (spherical equivalent ≤ -0.75 diopters) as a function of age for two birth cohorts (1910 to 1939, 1940 to 1979) with 95% confidence interval

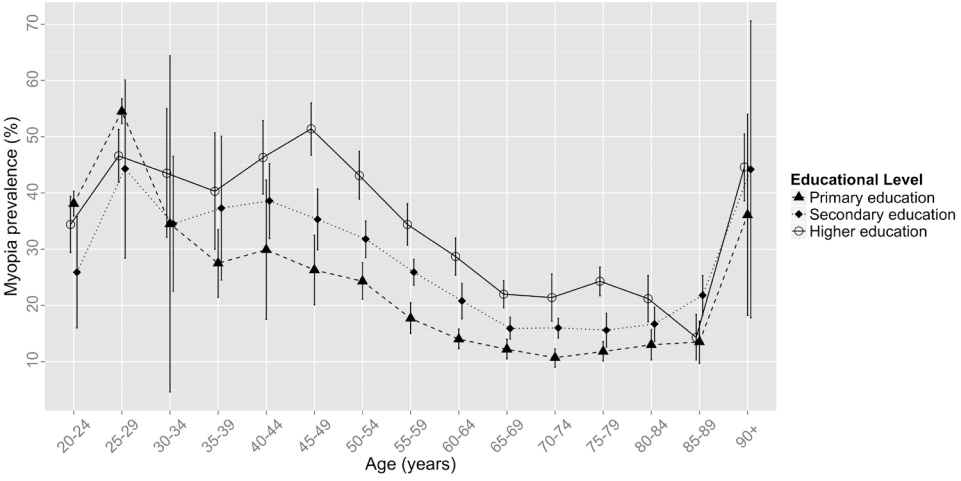

Figure 3. Prevalence of myopia (spherical equivalent ≤ -0.75 diopters) with 95% confidence intervals stratified by highest educational level achieved. Primary education - leaving education at <16 years old, secondary education - leaving school at ≤19 year of age, higher education - leaving school ≥20 years of age

was 26.3% (95% CI 20.1-32.5) with primary education compared to 51.4% (95% CI 46.7-56.0) with higher education, and in those aged 60-64 myopia prevalence was 14.0% (95% CI 12.3-15.8) compared to 28.7% (95% CI 25.4-32.0) for primary and higher education respectively. The trends observed are less clear in younger subjects (<35) in Figure 3, most likely due to small sample sizes (n=216 aged 20-25 years, n=336 aged 25-30 years), which are further stratified by education level with corresponding wide confidence intervals.

Levels of education throughout Europe have increased in the last 90 years (Figure 4). The proportion of individuals progressing to higher education rose from 4% in those born in the 1900s, to 16% in the 1920s, 20% in the 1940s, 33% in the 1960s and to approximately 61% in those born in the 1980s.

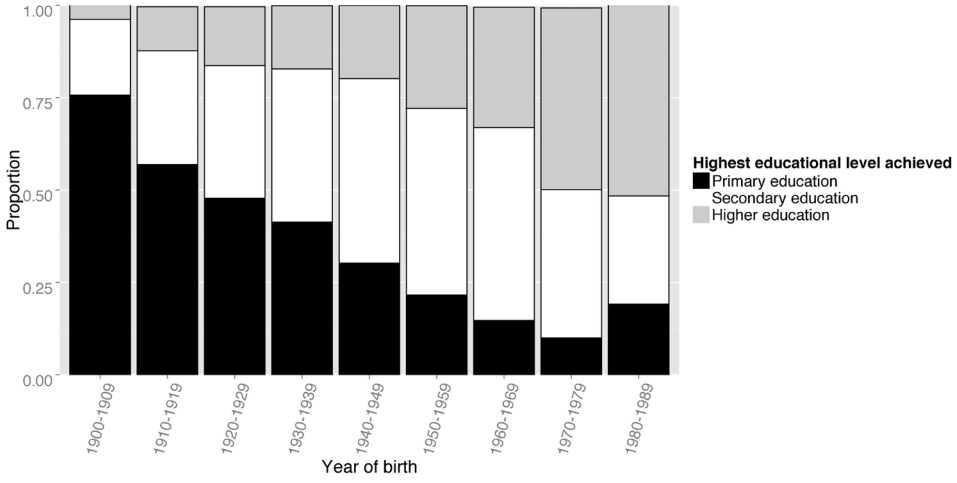

Figure 4. Distribution of highest educational level achieved, stratified by year of birth (1900-1989)
Primary education - leaving education at <16 years old, secondary education - leaving school at ≤19 year of age, higher education - leaving school ≥20 years of age

However, although those born more recently were more likely to have achieved a higher educational level, this alone did not explain the cohort effect of rising myopia. As shown in Figure 5, for individuals aged 45-65 years (age range selected for minimal age-related myopia variance and large available sample size), the increase in myopia prevalence with a more recent birth decade was observed across all educational groups. This was most pronounced for participants only achieving a primary education, where myopia prevalence increased from 10.7% (95% CI 7.6-13.8) to 28.1% (95% CI 18.1-38.0) between birth decades 1920-29 and 1960-69 (p=0.001). The corresponding increase in myopia in those with higher education was from 26.0% (95% CI 17.4-34.6) to 40.2% (95% CI 30.5-50.0) (p=0.03). Compared to the reference risk of participants with primary education and born in the 1920s, the myopia prevalence ratio for those achieving a higher education was 2.43 (95% CI 1.26-4.17) and 2.62 (95% CI 1.31-5.00) for those born in the 1960s. Individuals both born in the 1960s and completing higher education had almost four times the baseline risk, with a prevalence ratio of 3.76 (95% CI 2.21-6.57). Thus, the individual associations of educational level and birth cohort had an additive effect on myopia prevalence.

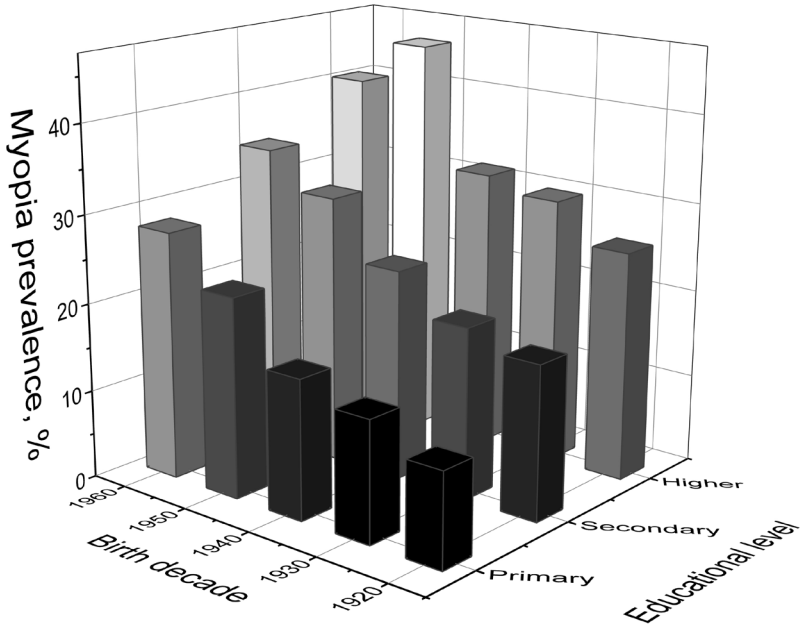

Figure 5. Myopia prevalence (spherical equivalent ≤ -0.75 diopters) by birth cohort and educational level in individuals aged 45-65 years old

Primary education - leaving education at <16 years old, secondary education - leaving school at ≤19 year of age, higher education - leaving school ≥20 years of age

DISCUSSION

Our study provides the first evidence that myopia is becoming more common across Western and Northern Europe, with a clear trend of higher myopia prevalence in participants with a more recent birth year (Figure 1). This is similar to the increase reported in North America and, albeit to a lesser extent, Southeast Asian populations[6,7,31,32]. Evidence of rising myopia prevalence carries clinical and economic implications. The increased requirement for detection and treatment of myopia, entailing glasses, contact lenses or more recently laser refractive surgery, has significant implications for clinical optometric and ophthalmic service provision, and the health care system. Additional ophthalmic services will be needed for treatable sight threatening complications such as retinal detachment, glaucoma and cataract[10,33]. The rising prevalence of myopia also implies that untreatable complications, such as myopic maculopathy, most commonly seen in high myopia, will become more common. This will result in more visual impairment in middle to late aged individuals, including a proportion of the working age population, with consequent economic implications.

Myopia has been strongly associated with education[2,21,24,34] and we explored this using a simple three-tier classification of educational level. Increasing educational level had a strong effect, with myopia twice as common in those achieving a higher education compared to participants leaving

school before 16. There was a clear trend of increasing prevalence of myopia across the tiers of education level, suggesting a potential additive effect of years of education. This interesting association may reflect a number of factors; greater near work activities with more education and less time in outdoor light, shared genetic factors underlying myopia and intelligence, or factors related to educational opportunity such as socioeconomic status or maternal nutrition. These associations have been explored in younger cohorts[18,20,21,35-37], although causal pathways are yet to be fully understood.

Reasons for the observed cohort effect are clearly multifactorial, and education is an obvious possible explanation; in our data only 12% of participants born in the 1920s went on to higher education, compared to 33% born in the 1960s. This educational expansion has been observed across Europe in both men and women, with a sharp trajectory towards mass higher education after World War II[28,38]. In addition to disruption of education and economic consequences of World War II, adverse health outcomes have been reported in young people growing up at that time, notably diabetes, depression and heart disease[39]. Whilst there is no known direct link between these health issues and myopia, the deprivation may have affected eye growth and resulting refraction. Certainly there was a rise in myopia in subjects born after 1950, but it is difficult to be certain what aspect of the seismic changes in Europe after the war might be responsible.

However, although the younger generations were more educated, we found a clear increase in the prevalence of myopia across the birth cohorts within each educational stratum as well as the additive effect of educational status. Therefore increasing levels of myopia were not explained by education alone and a more recent birth year and higher educational level had an additive effect on myopia risk. Our simple three-tier education stratification may be subject to residual confounding from variation in educational practices and it may be these, rather than changes in education level, that are contributing to the observed cohort effect. In the latter half of the last century, there have been increasing use of computers, increasing length of the educational day with increased after-school tuition, and less outdoor play as a result of reduced recess time[40].

The E^3 consortium has provided a large dataset to meta-analyse temporal trends and educational associations for myopia prevalence across Europe. Limitations to this consortium meta-analysis include heterogeneity between studies. Contributing studies inherently differed in study design and cohort sampling. Acknowledging this heterogeneity we have performed a random rather than a fixed meta-analysis, assuming no fixed effect between studies. There are also differences between European countries in terms of urbanization, economy, social class, education and lifestyle, which are known to influence myopia. Data on these variables at an individual or study-specific level was not uniformly available, and data collection was often performed in middle aged and older participants, so retrospective collection of potential contributing factors such as outdoor exposure, amount of reading and area of residence during the critical first 20 years of refractive error development would be impossible. Additionally, potential multicollinearity of these likely highly correlated factors (eg. reading and education) would make assessment of separate effects difficult. In attempt to reduce heterogeneity arising from these associated factors we performed a random effects meta-analysis stratified by age and educational level (both significantly associated

with myopia). Applicability of our findings is greatest for middle to older aged individuals and to those from Northern and Western European countries, given the sampled ages and the location of the E^3 studies (Table 1).

Further limitations include the crude nature in which education was classed, which as previously acknowledged may result in residual confounding. In addition, education status was collected retrospectively and therefore prone to recall error, possibly heightened in older participants. Refractions were all non-cycloplegic, although this is reasonable given the age of participants[41,42]. Finally, these data are not longitudinal, so we have not examined reasons for the lower prevalence with age within birth decades, although the cohort effect we have identified may be part of this explanation. Other reasons include the well-known hyperopic shift with age, and could include other factors such as censoring with age if myopic subjects have earlier cataract surgery.

We can conclude, for the first time, that the prevalence of myopia is increasing in Europe; a finding that is not fully explained by rising education levels despite higher educational achievement being associated with myopia and becoming more widespread in Europe. The changes in prevalence are similar to that observed in North America although remain far less than that identified in Southeast Asia, possibly due to differing intensity of education from an early age[1,6,40]. High levels of myopia were detected in the younger adults with a more recent birth year, of whom nearly half were affected. This has significant implications for the future; increasing myopia prevalence, and specifically high levels in younger individuals, will potentially result in an increasing burden of associated visual impairment in the future.

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4.2

Education influences the role of genetics in myopia

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ABSTRACT

Myopia is a complex inherited ocular trait resulting from an interplay of genes and environmental factors, most of which are currently unknown. In two independent population-based cohorts consisting of 5256 and 3938 individuals from European descent, we tested for biological interaction between genetic predisposition and level of education on the risk of myopia. A genetic risk score was calculated based on 26 myopia-associated single nucleotide polymorphisms recently discovered by the Consortium for Refractive Error and Myopia. Educational level was obtained by questionnaire and categorized into primary, intermediate, and higher education. Refractive error was measured during a standardized ophthalmological examination. Biological interaction was assessed by calculation of the synergy index. Individuals at high genetic risk in combination with university-level education had a remarkably high risk of myopia (OR = 51.3; 95% CI 18.5-142.6), while those at high genetic risk with only primary schooling were at a much lower increased risk of myopia (OR = 7.2, 95% CI 3.1-17.0). The combined effect of genetic predisposition and education on the risk of myopia was far higher than the sum of these two effects (synergy index 4.2, 95% CI 1.9-9.5). This epidemiological study provides evidence of a gene-environment interaction in which an individual's genetic risk of myopia is significantly affected by his or her educational level.

INTRODUCTION

Myopia (nearsightedness) is the most common refractive error and one of the leading causes of blindness[1,2]. Myopia currently affects more than one in four people in the United States and Western Europe[3], and has a prevalence higher than 70% in urban areas in Asian countries[4,5]. The global incidence of myopia is growing[6,7], increasing the frequency of sight-threatening complications such as myopic macular degeneration, glaucoma, and retinal detachment[8-10].

Myopia is highly heritable; the risk of developing myopia is increased at least three-fold among children with two myopic parents compared to children with no myopic parents[11,12], and heritability estimates for refractive error range from 0.60 to 0.90[13]. The Consortium for Refractive Error and Myopia (CREAM) and 23andMe independently conducted large genome-wide association studies, and identified more than 20 genetic loci for this trait[14-16]. Individuals with many risk variants at these loci have a tenfold increased risk of myopia[14].

Education is the most important environmental risk factor for myopia identified to date[17].The risk of developing myopia is up to four times higher in persons with a university-level education compared to persons with only primary schooling[17]. Achieving a higher level of education requires many hours of intensive near work (up-close work)—particularly reading—and this may contribute to the increased relative risk of developing myopia. Indeed, an increase in the average population-wide educational level may have contributed to the recent rise in the prevalence of myopia[6,7,18]. There are hints that education may influence the effect of myopia genes, e.g., a study of an Amish population found that the refractive errors of well-educated carriers of the *MMP1* and *MMP10* risk variants tended to be more myopic than those of individuals with lower levels of education[19]. Whether this gene-education interaction plays a role in the entire spectrum of genetic variants is unknown.

We assessed the combined effect of genetic predisposition and educational level on the risk of myopia in two independent population-based cohorts from Rotterdam, the Netherlands. We computed a genetic risk score based on 26 established loci for refractive error, calculated mean refractive error as a function of genetic risk score for levels of education, estimated risk of myopia in combined strata of genetic risk and educational level, and examined biological interaction according to the synergy index developed by Rothman[20].

MATERIAL AND METHODS

Study population

The study population consisted of participants from the Rotterdam Study cohorts who had baseline data on refractive error, educational level and genotype. All measurements were conducted after the Medical Ethics Committee of the Erasmus University had approved the study protocols and all participants had given a written informed consent in accordance with the Declaration of Helsinki. All participants were from European descent.

Rotterdam Study I (RS-I) was used as discovery cohort (Table 1). This prospective population-

Table 1. Characteristics of the study population from all cohorts

	Discovery cohort	Replication cohort		Combined
	RS-I	RS-II	RS-III	RS-I, RS-II, RS-III
N	5256	1984	1954	9194
Sex, % men (±SD)	42	46	44	43
Age, yrs (±SD)	68.4±8.5	64.2±7.5	59.1±5.5	64.9±9.2
Baseline examinations	1991-1993	2000-2002	2006-2009	1991-2009
Refractive error				
Mean refractive error, D (±SD)	0.85±2.45	0.47±2.51	-0.34±2.61	0.52±2.54
High myopia ≤-6D , %	91 (1.7)	35 (1.8)	61 (3.1)	187 (2.0)
Medium myopia >-6D & ≤-3D , %	268 (5.1)	145 (7.3)	240 (12.3)	653 (7.1)
Low myopia -3D & ≤-0.75D , %	500 (9.5)	258 (13.0)	358 (18.3)	1116 (12.1)
Emmetropia >-0.75D & <0.75D , %	1355 (25.8)	528 (26.6)	625 (32.0)	2508 (27.3)
Low hyperopia ≥0.75D & <3D , %	2309 (43.9)	813 (41.0)	549 (28.1)	3671 (39.9)
Medium hyperopia ≥3D & <6D , %	661 (12.6)	187 (9.4)	104 (5.3)	952 (10.4)
High hyperopia ≥6D , %	72 (1.4)	18 (0.9)	17 (0.9)	107 (1.2)
Educational level				
Primary education , %	2798 (53.2)	651 (32.8)	522 (26.7)	3871 (43.2)
Intermediate education , %	1850 (35.2)	912 (46.0)	807 (41.3)	3569 (38.8)
Higher education, %	608 (11.6)	421 (21.2)	625 (32.0)	1654 (18.0)
Genetic risk				
Mean genetic risk score (±SD)	2.7±0.4	2.7±0.4	2.7±0.4	2.7±0.34
Low genetic risk score (1.40-2.25), %	463 (8.8)	173 (8.7)	164 (8.4)	800 (8.7)
Mean N carried risk alleles (±SD)	17.7±1.4	17.6±1.4	17.6 (1.5)	17.7±1.4
Medium genetic risk score (2.25-3.00), %	3582 (68.2)	1364 (68.8)	1334 (68.3)	6280 (68.3)
Mean N risk alleles (±SD)	22.7±1.9	22.8±2.0	22.7 (1.9)	22.8±1.9
High genetic risk (3.00-4.00) , %	1211 (23.0)	447 (22.5)	456 (23.3)	2114 (23.0)
Mean N risk alleles (±SD)	27.7±1.7	27.7±1.7	27.7±1.7	27.7±1.7

Values are means ± standard deviation.
SD, standard deviation; RS, Rotterdam Study; D; diopters.

based cohort study included a total of 5256 participants aged 55 years and older living in Ommoord, a suburb of Rotterdam, the Netherlands[21]. Baseline examinations took place between 1991 and 1993. Two independent Rotterdam Study cohorts were combined into a replication cohort (Table 1). The first cohort was Rotterdam Study II (RS-II), an independent cohort which included n = 1984 participants aged 55+ years living in Ommoord since 2000[21]. Baseline examinations took place between 2000 and 2002. The second cohort was Rotterdam Study III (RS-III), which included n = 1954 participants aged 45+ years and older living in Ommoord since 2006[21] .Baseline examinations took place between 2006 and 2009.

Assessment of refractive error

All participants underwent a complete ophthalmological examination including a non-dilated

measurement of refractive error of both eyes using a Topcon RM-A2000 autorefractor. Refractive error was analyzed as spherical equivalent, calculated according to the standard formula 'SE = sphere + ½ cylinder'. Mean refractive error was calculated; when data from one eye was unavailable, the SE of the other eye was used. Exclusion criteria were (bilateral) cataract surgery and laser refractive procedures without knowledge of prior refraction, other refraction influencing intra-ocular procedures, keratoconus, and syndromes. Refractive error was categorized into high myopia (≤ -6 diopters (D)), moderate myopia (>-6D & ≤-3D), low myopia (<-3D & ≤-0.75D), emmetropia (> -0.75D & <0.75D), low hyperopia (≥0.75D & <3D), medium hyperopia (≥ 3D & <6D), and high hyperopia (≥ 6D), using criteria defined by the CREAM consortium (CREAM consortium meeting, 2012, Sardinia, Italy).

Assessment of educational level

Information on educational level was obtained during a home interview. Level of education was classified into: primary education (primary school or lower vocational education); intermediate education (lower secondary education or intermediate vocational education); and higher education (higher secondary education, vocational education, or university).

Genotyping

We selected all 26 genome-wide significant single nucleotide polymorphisms (SNPs) associated with refractive error and myopia derived from a meta-analysis from the CREAM consortium involving a total of 45,758 study subjects[14]. SNP genotyping and imputation have been described in detail elsewhere[22]. Genotyping was performed using the Illumina Infinium II HumanHap550 chip v3.0 array (RS-I); the HumanHap550 Duo Arrays and the Illumina Human610-Quad Arrays (RS-II), and the Human 610 Quad Arrays Illumina (RS-III). For imputation, we used the Markov Chain Haplotyping (MACH) package version 1.0.15 software (imputed to plus strand of NCBI build 36, HapMap release #22, CEU panel). Most of the SNPs were genotyped or had a high imputation quality score (r2 ≥ 0.8)**.**

Genetic risk score

The genetic risk score was calculated based on all 26 SNPs using a previously reported weighting method[14]. Each SNP was weighted according to its relative effect size (β regression coefficient from CREAM meta-analysis, Supplementary Table 2). Genetic risk scores ranged from 1.4 to 4.0, with higher scores indicating a greater genetic predisposition to myopia. The genetic risk score was categorized into a low (1.4-2.25), medium (2.25-3.00) or high genetic load (3.00-4.00) based on the association with myopia (Supplementary Figure 1). We also calculated the number of risk alleles carried per individual (homozygote for the risk allele = 2 risk alleles, heterozygote = 1 risk allele, homozygote for the other allele = 0 risk alleles).

Statistical analysis

Separate analyses were performed for the discovery cohort (RS-I), the replication cohort (RS-II and RS-III combined), and for the cohorts combined (RS-I, RS-II, and RS-III). First, we assessed independent associations between education and refractive error and myopia, and genetic risk score and refractive error and myopia using linear and logistic regression. Second, we examined

the continuous relation between genetic risk score, level of education and refractive error by calculating mean refractive error and the regression coefficients β per genetic risk score category, stratified by level of education, and tested for significant differences between groups with a one way ANOVA F-test. Third, we assessed the risk of moderate to high myopia (refractive error ≤ -3.0 D) versus moderate to high hyperopia (refractive error ≥ 3.0 D) for combined strata of genetic risk score and educational level with logistic regression analyses, using low genetic risk score and primary education as the reference, adjusting for age and sex. These analyses were also performed using moderate to high myopia (refractive error ≤ -3.0 D) versus emmetropia (refractive error > -0.75D & <0.75D) as the outcome.

We tested for biological interaction between genetic predisposition and education by calculating the age and sex adjusted synergy index (SI) according to Rothman[20]. This measures deviation from additivity of 2 factors, and is based on the ratio of the combined effect to the sum of the separate effects. A synergy index of more than 1.0 suggests that the effect of both factors together is greater than the sum of the effect of the separate factors.

All reported *P* values are nominal and two-sided. We used SPSS version 20.0.0 (SPSS Inc.) for all analyses.

RESULTS

Demographics of the study participants in the discovery (RS-I) and in the replication (RS-II and RS-III combined) cohorts can be found in Table 1. In all cohorts, the majority of subjects were low hyperopic or emmetropic; the mean refractive error was 0.52 D (SD 2.54). Primary or intermediate educational level was most common, although its relative proportion was highest in the discovery cohort (RS-I) (Table 1). The genetic risk score ranged from 1.4 to 4.0 with a mean of 2.7 (SD 0.4), corresponding to a range of 12 to 35 carried risk alleles, and a mean of 23.4 (SD 3.3) risk alleles per subject. The genetic risk score had identical distributions across all cohorts (Table 1). Both educational level and the genetic risk score were significantly associated with refractive error and myopia (P<0.0001, Table 2).

The continuous relation between genetic risk score and refractive error stratified by level of education for the combined cohorts is shown in Figure 1. Subjects who received a university or higher vocational education had a lower mean refractive error with increasing genetic risk than subjects with intermediate-level or primary education. These differences were statistically significant ($\beta_{\text{high education}}$ = -0.78; $\beta_{\text{intermediate}}$ = -0.53; β_{primary} = -0.47; P<0.0001 for both the discovery and replication cohorts). Among individuals with the highest genetic risk, the refractive error averaged -2 diopters for high educational level, -0.8 diopters for intermediate education, and 0 diopters or emmetropia for primary schooling.

We then estimated the risk of myopia for the combined strata of genetic risk and educational level (Table 3, Figure 2). In both the discovery and replication cohorts, the risk of myopia among subjects with a high genetic risk score and high educational level was highly increased (OR_{combined} = 51.3; 95% CI: 18.5-142.6), and far higher than the sum of the risks among individuals with only

Table 2. Association with refractive error and risk of myopia for genetic risk score and level of education

	Refractive error				Myopia			
	n	β	se	P-value	n	OR	95% CI	*P-value*
Education								
Discovery cohort (RS-I)	5256	-0.48	0.05	<0.0001	1092	2.3	1.9-2.8	<0.0001
Replication cohort (RS-II & RS-III)	3938	-0.58	0.06	<0.0001	807	2.2	1.7-2.7	<0.0001
Combined (RS-I, RS-II, RS-III)	9194	-0.55	0.04	<0.0001	1899	2.3	12.0-2.6	<0.0001
Genetic risk score								
Discovery cohort (RS-I)	5256	-0.67	0.06	<0.0001	1092	2.4	1.9-3.1	<0.0001
Replication cohort (RS-II & RS-III)	3938	-0.72	0.07	<0.0001	807	3.1	2.3-4.2	<0.0001
Combined (RS-I, RS-II, RS-III)	9194	-0.69	0.05	<0.0001	1899	2.7	2.2-3.2	<0.0001

Beta regression coefficients of the association with refractive error were calculated using linear regression analyses. The risk of myopia (defined as refractive error ≤-3 diopters) were calculated using logistic regression analyses with hyperopia (defined as a refractive error ≥3 diopters) as a reference. Analyses for education were corrected for age, sex, and genetic risk score. Analyses for the genetic risk score were corrected for age, sex, and education. β, beta regression coefficient in diopter; se, beta standard error; OR, odds ratio; 95% CI, 95% confidence interval; RS, Rotterdam Study.

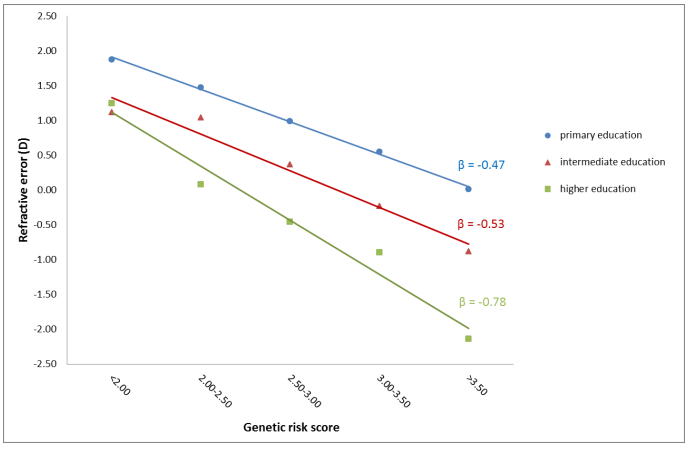

Figure 1. Refractive error as a function of genetic risk score stratified by level of education
Mean refractive error was calculated for each genetic risk score category and presented according to educational level. Regression lines were plotted, and the regression coefficient (β) is indicated for each line. The data are shown for the combined cohort (including RS-I, RS-II, and RS-III). The differences between educational level groups were statistically significant (P<0.0001) for the discovery, replication and combined cohorts.

one of these two factors ($OR_{combined}$ for primary education = 6.1, 95% CI: 2.1-17.6.; $OR_{combined}$ for high genetic risk = 7.2, 95% CI: 3.1-17.0).

The synergy index according to Rothman[20] was statistically significant in both the discovery cohort and the replication cohort ($SI_{combined}$ = 4.2; 95% CI: 1.9-9.5), indicating a biological interaction (Table 3).

Table 3. Risk of myopia for educational level and genetic risk score, adjusted for age and sex

	primary education			intermediate education			higher education			P value for trend
Discovery cohort, RS-I (n = 1092)	**n**	**OR**	**95% CI**	**n**	**OR**	**95% CI**	**n**	**OR**	**95% CI**	
low genetic risk	65	1.0 (R)	NA	42	4.3	1.1-17.1	14	5.9	1.1-30.9	0.001
medium genetic risk	386	4.6	1.4-15.1	268	9.1	2.7-29.9	88	23.5	6.7-82.2	<0.0001
high genetic risk	105	8.4	2.4-28.9	93	26.5	7.6-91.5	31	71.6	15.6-328.3	<0.0001
SI 5.5; 95% CI 1.6-18.5										

	primary education			intermediate education			higher education			P value for trend
Replication cohort, RS-II & RS-III (n = 807)	**n**	**OR**	**95% CI**	**n**	**OR**	**95% CI**	**n**	**OR**	**95% CI**	
low genetic risk	23	1.0 (R)	NA	24	0.7	0.1-3.8	20	5.5	1.3-23.4	0.04
medium genetic risk	140	2.8	0.8-8.9	233	4.6	1.5-14.3	164	14.6	4.5-47.3	<0.0001
high genetic risk	50	7.5	2.1-26.1	92	19.0	5.6-64.8	61	37.2	9.1-152.3	<0.0001
SI 3.3; 95% CI 1.1-9.9										

	primary education			intermediate education			higher education			
Combined cohorts, RS-I, RS-II, RS-III (n = 1899)	**n**	**OR**	**95% CI**	**n**	**OR**	**95% CI**	**n**	**OR**	**95% CI**	**P value for trend**
low genetic risk	88	1.0 (R)	NA	66	2.0	0.7-5.5	34	6.1	2.1-17.6	0.008
medium genetic risk	526	3.5	1.5-7.9	501	6.4	2.9-14.4	252	18.8	8.1-43.7	<0.0001
high genetic risk	155	7.2	3.1-17.0	185	21.6	9.2-50.6	92	51.3	18-5-142.6	0.007
SI 4.2; 95% CI 1.9-9.5										

Myopia was defined as a refractive error ≤-3 diopters. For this analysis, subjects with hyperopia (defined as refractive error ≥3 diopters) were used as controls.
OR, odds ratio; 95% CI, 95% confidence interval; SI, synergy index; RS, Rotterdam Study; R, reference; NA, not applicable.

The risks in the combined strata using myopia versus emmetropia as the outcome showed similar trends, however, ORs were lower in all strata and the synergy index did not reach statistical significance (Supplementary Table 1, Supplementary Figure 2).

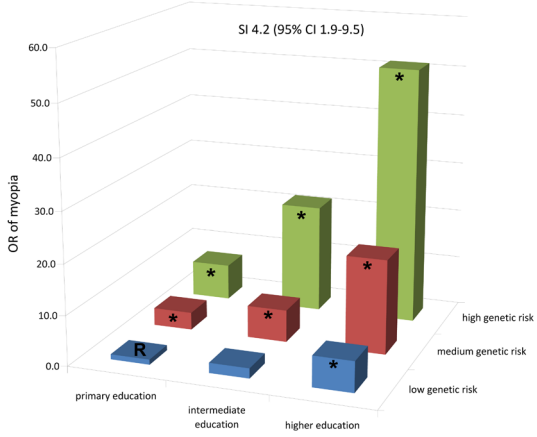

Figure 2. Risk of myopia for educational level and genetic risk score
The age- and sex-adjusted odds ratio for myopia (defined as a refractive error ≤-3 diopters) versus hyperopia (defined as a refractive error ≥3 diopters) for educational level and genetic risk score are plotted for the combined cohort (including RS-I, RS-II, and RS-III). The group with low genetic risk and primary education served as the reference.
*, significant OR compared to the reference group; SI, synergy index; 95% CI, 95% confidence interval; OR , odds ratio; R, reference (i.e., OR = 1.0).

DISCUSSION

In two independent cohorts from the population-based Rotterdam Study, we found a significant biological interaction between education and genetic risk of myopia as represented by 26 associated SNPs[14]. Subjects with high genetic risk in combination with high levels of education had a far higher risk of myopia than subjects with only one of these two factors. We observed this effect in both quantitative analyses with refractive error in diopters as a continuous outcome, as well as in qualitative analyses comparing the extreme ends of the physiological spectrum. The interaction effect of genetic predisposition and education on myopia risk was more than 4 times higher than the sum of the separate effects.

Our study has specific strengths. First, the size of the combined study population and the frequency of exposures and outcomes were sufficiently high to detect a biological interaction. In addition, the interaction and the risk estimates were significant in the discovery cohort and were confirmed in the replication cohort, suggesting high reliability of these results. On the other hand, our study was limited by the rough approximations of the two risk factors (genetic risk and education level). Our genetic risk score was based on 26 myopia risk SNPs which were identified by the CREAM consortium, and of which 14 were also found by 23andMe[15]. The effect sizes of the

remaining 8 23andMe top hits were very small (betas between 0.03 to 0.08), and incorporation of these SNPs did not change our findings. Nevertheless, more in-depth knowledge regarding the genetic background of myopia in the future will improve precision of the effect sizes. In addition, education may be an even stronger effect modifier when absolute years of education can be incorporated. Finally, we observed a cohort effect that merits mention. Subjects from the RS-I study (which covered the period 1991 through 1993 and included subjects age 55 years and older) generally had a lower educational level than subjects from the RS-III study (which covered the period from 2006 through 2009 and included subjects age 45+ years). However, because the interaction effect of education and genetic risk was detected independently in each of these cohorts, this cohort effect did not likely affect our findings.

What mechanisms might underlie this strong interaction between education and genetic risk? Achieving higher levels of education requires more intensive near work. Several studies have reported that near work is directly related to the development of myopia by causing retinal defocus and degradation of retinal image contrast, which can subsequently trigger eye growth as a compensatory mechanism[23-27]. However, others point out that persons with a higher educational level are at risk of myopia because they spend less time outside[28]. Education may reflect a complex combination of these factors, ultimately leading to up-regulation of risk genes, excessive eye growth and development of myopia.

The 26 recently discovered SNPs are present in genes involved in various processes, including neurotransmission, ion channel function, extracellular matrix formation and stabilization, retinoic acid metabolism, and ocular development. As with gene-environment interactions described for other disorders[29], it is unlikely that all of these genes contribute to the gene-education interaction in myopia. We hypothesize that neurotransmission-related genes that are expressed in the outer retina may be particularly vulnerable to the effect of retinal defocus, in contrast to developmental eye genes and genes involved in the extracellular matrix. A genome-wide analysis of SNP-education interaction in a large study population might reveal the modifying effects of individual SNPs.

Interestingly, a combined effect between near work and outdoor activity —a known protective factor against myopia—has also been reported[28]. In addition, several studies have reported that outdoor activity can counteract the increased risk from near work[28,30,31]. Whether this type of work can also reduce the risk of near work among individuals at high genetic risk is an interesting question that merits investigation.

Genetic research regarding myopia has traditionally been guided by the assumption that genes exert a direct effect on the trait. Our finding of a robust gene-environment interaction casts new light on the current evolutionary model and offers new opportunities to identify additional myopia genes. Working many hours at near work tasks appears to be the requisite trigger for eliciting strong gene effects, and once this condition is satisfied, the genes become highly penetrant. We recommend that the search for new myopia genes should focus on study participants who are selected based on exposure (i.e., subjects with a high level of education and/or intensive near work work). This approach can also be readily extended to the study of other complex disorders.

If environmental exposures show considerable variation within the study sample, genes might account for only a small percentage of the phenotypic variation. However, if these exposures have low variability among the study cohort, a disease that had previously been believed to arise from many small genetic effects might actually be caused by only a few genes, each of which exerts a relatively large effect.

Traditionally, analyzing gene-environment interactions has been extremely challenging, and this is primarily because the low relative frequencies of the exposures and/or trait have limited the study's statistical power[32]. However, given that our analysis has overcome these limitations, this approach may serve as a textbook example of biological interactions between genes and the environment.

This epidemiological study provides evidence of gene-by-environment (GxE) interaction, in which an individual's genetic risk of myopia is affected by his or her educational level. Subjects with many variants in myopia genes and a higher educational level (e.g. university) are much more susceptible to develop myopia than those with only one of these two factors. Education may reflect a complex combination of higher level of reading exposure and corresponding lower levels of outdoor physical activity, ultimately leading to up-regulation of risk genes, excessive eye growth and the development of myopia.

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4.3

Meta-analysis of gene-environment-wide association scans accounting for education level identifies additional loci for refractive error: The CREAM consortium

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ABSTRACT

Myopia is the most common human eye disorder with complex genetic and environmental causes. The recent rapid rise in myopia prevalence globally poses a major public health challenge. We hypothesized that integrating educational information and its potential interaction with genetic variants may facilitate the detection of susceptibility genes for myopia, thus accounting for a greater proportion of its heritability. We surveyed common genetic variants across the genome in 40,036 adults from 25 studies of European ancestry and 10,315 adults from 9 studies of Asian ancestry and performed a joint meta-analysis to test main and SNP × education interaction effects on refractive error. In European ancestry individuals and combined cohorts, six novel genetic loci (*FAM150B*, *LINC00340, FBN1, DIS3L-MAP2K1, ARID2* and *SLC14A2*) associated with refractive error were identified ($P < 5.0 \times 10^{-8}$). In Asian populations, three genome-wide significant genetic variants highlighted genes *AREG*, *GABRR1* and *PDE10A* ($P < 5.0 \times 10^{-8}$), all of which showed strong interactions with education ($P < 8.5 \times 10^{-5}$). In support of a role for *GABRR1*, its expression was found to be upregulated in sclera and retina in a mouse myopia model, implicating neurotransmitter GABA signaling in myopia development. The discovery of these novel loci represents an important advance in the understanding how gene and environment interactions contribute to the heterogeneity of myopia.

INTRODUCTION

Myopia, or nearsightedness, has rapidly emerged as a global health concern in the last three decades[1]. It is one of the leading causes of visual impairment, and it is associated with potentially blinding ocular complications including retinal detachment, myopic maculopathy, glaucoma and cataract[2]. Evidence from family and twin studies strongly supports the heritability of myopia[3]. Estimates for the heritability of the quantitative trait refractive error have been reported to be as high as 90%[4]. On the other hand, the rapid upsurge of myopia in the last few decades in many parts of the world is likely to be a consequence of lifestyle changes, such as the increasing educational intensity, particularly in urban East Asia[5,6].

Major attempts undertaken in genome-wide association studies (GWAS) to elucidate the genetic determination of myopia and refractive error have recently led to the discovery of more than 30 *distinct susceptibility* loci[7,8]. Nevertheless, collectively these genetic variants explain only 5% of phenotypic variance[8]. As myopia is a result of the combination of genetic and environmental factors, interplay between genes and environment may account for a substantial proportion of the phenotypic variance[9]. Recently, we showed biological interactions between education and genetic risk score of myopia derived from 26 known GWAS SNPs in the Rotterdam Study[10]; the combined effect of genetic predisposition and education on the risk of myopia was substantially greater than anticipated from a simple sum of these two factors. At a gene level, a few genes, such as *DNAH9* which modulates neurotransmission between retinal cells, have been shown to interact with education level and exhibit strong genetic effects for myopia among Asians of at least higher secondary education[11]. In the current study, we hypothesized that genes implicated in myopia development may be uncovered by taking into account the potential interaction between genetic variants and education level.

In the context of the etiology of refractive errors, education level has largely been considered a surrogate measure for accumulated near work activity[1]. When viewing near objects, the eye generates extra optical power through the process of accommodation to focus the image on the on the retinal plane to maintain clear vision[12]. The retina has a central role in the mechanism linking such visual input with eye growth and refractive development[13]. Several neurotransmitters or moleculars have long been implicated in this process from animal studies including dopamine, acetylcholine, vasoactive intestinal peptide (VIP), gamma-aminobutyric acid (GABA) and glucagon[14,15]. However, an organized framework for the retinal signaling mechanisms underlying refractive error development under various environmental conditions remains to be elucidated.

Accounting for differences in environmental exposures may enhance power for the detection of genes, especially in circumstances where a genetic locus has a differential effect conditional on specific environment exposures[16,17]. Gene-environment-wide interaction studies (GEWIS) using a joint meta-analysis approach on SNP effects and SNP x environment interactions have recently been described[18,19]. This approach has proven successful in identifying six novel loci associated with fasting insulin and glucose accounting for interactions with body mass index[19]. It also led to the identification of two novel loci for pulmonary function that did not emerge from analyses based on the genetic main effects alone[20]. The well-documented effects of educational attainment on

myopia and refractive error make the proposed interaction an excellent analytical candidate for the GEWIS.

Availability of large-scale GWAS datasets on spherical equivalent from the Consortium for Refractive Errors And Myopia (CREAM) makes gene and environment (G × E) interaction analyses feasible. To identify additional genetic variants for refractive error, we performed GEWIS approach comprising 40,036 adults from 25 studies of European ancestry and 10,315 adults from 9 studies of Asian ancestry in the CREAM. Furthermore, we validated the relative over-expression of gamma-aminobutyric acid (GABA) receptor *GABRR1* in the retina and scleral tissues in myopic eyes in a mouse model.

MATERIAL AND METHODS

Study populations

From the Consortium of Refractive Error and Myopia (CREAM), A total of 34 studies comprising 40,036 individuals of European ancestry from 25 studies and 10,315 individuals of Asian ancestry from 9 studies were included for this analysis (Table 1; Supplementary Table 1-2). Individuals aged less than 20 years were excluded, as well as those who had undergone cataract surgery, laser or other intra-ocular procedures that could alter refraction. Many of these studies were also included in the previous CREAM GWAS on spherical equivalent[8]. All studies adhered to the tenets of the Declaration of Helsinki and were approved by their local research ethics committees. All participants provided a signed, informed consent before the start of the study.

Phenotyping and education levels

Participants in the included studies underwent an ophthalmological examination (Supplementary Table 1). Non-dilated refraction was measured by auto-refraction and/or subjective refraction. Spherical equivalent was calculated as the sphere power plus half of the cylinder power for each eye. The mean spherical equivalent of the right and left eyes was used as a quantitative outcome. When data from only one eye was available, the spherical equivalent of that eye was used. For education, subjects reported the highest level of education achieved, or the years of schooling through a self-reported questionnaire or in an interview.

We dichotomized education for all participants. The higher education group consisted of those who had completed at least higher secondary education, gained a polytechnical school certification, or with ≥ 12 years spent in formal education. The lower education group included individuals who had only completed lower secondary education or less, or with < 12 years of formal education. For four cohorts of relatively young European participants (BATS, DCCT, RAINE and WESDR; total sample size of 2,349), almost all of them had completed 12 or more years of schooling. We thus chose to categorize individuals with tertiary or university education as the higher education group in these studies. Sensitivity analysis excluding these four cohorts did not appreciably change our meta-analysis results (data not shown).

Genotyping and imputation

Detailed information on the genotyping platforms and data cleaning procedures for each study is provided in Supplementary Table 2. Each study applied stringent quality control filters for GWAS. In general, individuals reflecting duplicates, low call rate (< 95%), gender mismatch, or population outliers were excluded. SNPs were excluded if low genotyping call rate (> 5% missingness), monomorphic SNPs, with MAF < 1%, or in Hardy-Weinberg disequilibrium (p-value < 10^{-6}). After quality control (QC) filtering, the array genotypes of each study were imputed using the 1000 Genomes Project data[21] as reference panels (build 37, phase 1 release, March 2012) with the software Minimac[22] or IMPUTE[23] (Supplementary Table 2). SNPs which passed imputation quality thresholds (MACH: $r^2 > 0.5$ or IMPUTE info score > 0.5) and with minor allele frequency ≥ 5% were eligible for the meta-analysis.

Statistical models

For each study, a linear regression model at each genotyped or imputed SNP was constructed with the mean spherical equivalent as the outcome. We assumed an additive genetic model where the number of risk alleles is an ordinal variable (0, 1 and 2) for directly genotyped SNPs, or a continuous variable of allele dosage probability ranging from 0 to 2 for imputed SNPs. The primary analytic model included SNP, education, a SNP × education interaction term, as well as age and sex as covariates. Additional adjustments for the top principal components of genomic marker variations were performed in individual studies when applicable (*i.e.*, when there was evidence of population stratification).

We used the following additive genetic model to test for a joint effect of SNP (β_{SNP}) and SNP x education interaction ($\beta_{SNP \times education}$) on mean spherical equivalent: $Y = \beta_0 + \beta_{SNP} \times SNP + \beta_{education} \times Education + \beta_{SNP \times education} \times SNP \times Education + \beta_C \times Cov + \varepsilon$ (Model 1), where Y is the mean spherical equivalent, education is a dichotomous variable (0 = lower education group and 1 = higher education group); cov is a set of covariates such as age, sex and first top five principal components when applicable. For family-based studies, the kinship matrix was estimated empirically from the SNP data and included as a random effect in the generalized mixed model[24]. To test an effect of SNP x education interaction, we assessed $\beta_{SNP \times education}$ from Model 1.

The linear regression analyses in each study were conducted with Quickest (http://toby.freeshell.org/software/quicktest.shtml) or ProbABEL[25] for the unrelated samples, and MixABEL[24] for family-based data. The command 'robust' was used in the above software to calculate the robust ('sandwich', Huber-White) standard errors of β_{SNP} and $\beta_{SNP \times education}$, and error covariance of βs, to correct the potential inflation of false positive rate for the interaction p-value[26].

In addition, each study also tested the main effect of education on spherical equivalent by adjusting for age and gender using the linear regression model: $Y = \beta_0 + \beta_{education} \times education + \beta_C \times Cov + \varepsilon$ (Model 2), where the definition of variable is the same as in Model 1.

GEWIS join meta-analyses

We adopted the joint meta-analysis (JMA) approach[18,27] to simultaneously test both main SNP effects and SNP × education interactions for spherical equivalent with a fixed-effect model, using

SNP and SNP × education regression coefficients and a betas' covariance matrix from each study. A Wald statistic, following a chi-square distribution with two degrees of freedom, was used to test the joint significance of the SNP and SNP × education regression coefficients. The JMA was performed with METAL[28], using a script patch provided by Manning *et al*[27]. A Cochran's Q test was used to assess heterogeneity of the beta coefficients across studies for the SNP and interaction effects. To test for interaction between the SNP and education, we conducted a secondary meta-analysis of the SNP × education interaction effects for spherical equivalent (one degree of freedom) with a fixed-effects model using inverse-variance weighting in METAL; this is a traditional meta-analysis to investigate SNP × education interactions *per se*. Effects and standard error of the SNP (β_{SNP}) on spherical equivalent in the lower education group and higher education ($\beta_{SNP} + \beta_{SNP \times education}$) were derived from the JMA output[27].

We performed a meta-regression to explore sources of heterogeneity in our meta-analysis for three loci showing G x E interactions (R package 'metafor'). Meta-regression included the following study-specific variables as covariates: study sample size, proportion of individuals in the higher education group, average spherical equivalent, education main effects, ethnicity, study design, study year, and average age. Meta-regression was also conducted to test the fold-changes of the interaction beta coefficients in Asians versus Europeans for the 39 known myopia loci.

The study-specific genomic control inflation factors λ_{gc} for the joint test for SNP and interaction term ranged from 1.009 to 1.125 with an average of 1.019 (Supplementary Table 2), calculated by the ratio of the observed median chi-square divided the expected median of the 2df chi-square distribution (1.382). Genomic control (GC) correction was applied to chi-square statistics in each individual study[29]. For three studies of small sample sizes (N < 500) and λ_{gc} greater than 1, we further, prior to starting the meta-analysis, excluded SNPs showing significant joint *P* value < 1×10^{-5} but neither the main effects nor the interaction effects supporting such an association. Quantile-quantile (QQ) plots of the *p*-values showed only modest inflation of the test statistics in the JMA test (Europeans: $\lambda_{gc} = 1.081$; Asians: $\lambda_{gc} = 1.053$; Combined: $\lambda_{gc} = 1.092$; Supplementary Figure 1), similar to previous GEWIS studies with comparable sample sizes[19,20]. We excluded a small number of markers in the meta-analysis with $P_{HET} < 0.0001$. The λ_{gc} for the SNP x education interaction term in the individual studies ranged from 1.01 to 1.08, indicating little evidence of test statistic inflation for each study.

Annotation of genetic variants

The coordinates and variant identifiers are reported on the NCBI B37 (hg19) genome build, and annotated using UCSC Genome Browser[30]. We identified variants within each of the linkage disequilibrium (LD) blocks ($r^2 \geq 0.8$) in European and Asian populations of the 1000 Genomes Project (100 Kb flanking the top SNP; hg19) to apply functional annotations with experimental evidence of transcription regulation using HaploReg[31] and Encyclopedia of DNA Elements (ENCODE)[32] data.

Animal procedures

Differential gene expression

We further evaluated gene expression of GABRR1 using a mouse model of myopia. Experimental myopia was induced in B6 wild-type (WT) mice (n = 36) by applying a -15.00 D spectacle lens to the right eye (experimental eye) for 6 weeks from post-natal day 10[33,28]. The left eyes were uncovered and served as contra-lateral controls. Age-matched naive mice eyes were used as independent control eyes (n = 18). Each eye was refracted weekly using an automated infrared photo refractor as described previously[29]. Animal study approval was obtained from the SingHealth Institutional Animal Care and Use Committee (AAALAC accredited). All procedures performed in this study complied with the Association of Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmology and Vision Research.

Eyes were enucleated after six weeks of myopia induction and retina was carefully dissected out. RNA extraction, real-time polymerase chain reaction (qRT-PCR) methods, and analysis were followed as described previously[28]. qRT-PCR primers were designed with ProbeFinder 2.45 (Roche Applied Science) and performed with a Lightcycler 480 Probe Master (Roche Applied Science). The primer sequences were as follows: *GABRR1* Forward: tgcctgctagagtcccctta and Reverse: ccgtgatgatggtggacat. The experiments were repeated in triplicate. Mean values from the triplicates were used in the statistical analysis. Student's t-test was performed to determine the significance of the relative fold difference of mRNA between the myopic eyes of the experimental mice and the age-matched controls.

Immunohistochemistry and Western blot

Protein expression and localization were assessed by immunohistochemistry and Western blot. Whole mouse eyes (n = 6) were enucleated and embedded in Optimal Cutting Temperature compound at -20 °C for 1 hour. Six-micron sections were cut with a cryostat (HYRAX C 50, Carl Zeiss Microimaging GmbH, Germany) and collected on POLYSINE™ microscope glass slides (Gerhard Menzel, Thermo Fisher Scientific, Newington, CT). Sections were air dried at room temperature for 1 hour. The procedure for immunofluorescence staining has been described previously[28]. Immunofluorescent staining using specific antibodies for *GABRR1* (Ab85667; Abcam [Cambridge, MA, USA]) was carried out in the mouse myopic retina, choroid and scleral tissues to study the localization of these proteins. Sections incubated with 4% BSA without primary antibody were utilized as negative controls. A fluorescence microscope (Axioplan 2; Carl Zeiss Meditec GmbH, Oberkochen, Germany) was used to examine the slides to capture image. Experiments were repeated in duplicate from two batches (3 eyes per batch). We performed a western blot on retinal samples to quantify the protein expression in myopic eyes and naive control eyes. Protein extraction and quantification was carried-out as described previously[28].

Gene expression in human tissues GWAS Meta-analyses and SNP functional annotation

To assess gene expression in human tissues, we examined the Ocular Tissue Database and the EyeSAGE database[34,35]. The estimated gene and exome level abundances are available online (https://genome.uiowa.edu/otdb). Normalization of gene expression used the Probe Logarithmic Intensity Error (PLIER) method with GC-background correction[34].

RESULTS

Educational level and its main effects on spherical equivalent

Baseline characteristics of 50,351 participants from 34 studies in our meta-analysis are shown in Table 1. A total of 40,036 of subjects were of European ancestry and 10,315 were of Asian ancestry; the age of the participants ranged from 20 to 99 years. Among Europeans, the proportions of participants who completed higher secondary education ranged from 16.0% (FITSA and OGP Talana) to 94.4% (AREDS) with an average of 50.7% (Supplementary Table 1). In Asians, the proportions of individuals who completed higher secondary education ranged from 6.7% (SiMES) to 75.9% (Nagahama) with an average of 30.0%. Across all studies, individuals in the higher education group had a spherical equivalent refractive error that was on average 0.59 diopters (D) more myopic, or less hyperopic, compared to those in the lower education group (β = -0.59; 95% CI: -0.64, -0.55). High education level was associated with a two-fold more myopic spherical equivalent in individuals of Asian as compared to European ancestry (Asians: β = -1.09, 95% CI: -1.20, -0.98; Europeans: β = -0.49, 95% CI: -0.54, -0.44; Figure 1).

GEWIS in Europeans

The genome-wide joint meta-analysis (JMA) for SNP main effect and SNP × education interaction in 40,036 European Ancestry individuals showed association with spherical equivalent at 12 previously implicated loci (Figure 2A & Supplementary Table 3). We also identified 4 previously unreported loci associated with spherical equivalent achieving genome-wide significance ($P_{JMA} < 5.0 \times 10^{-8}$; $P_{het} \geq 0.086$; Table 2): *FAM150B, LINC00340, FBN1,* and *DIS3L-MAP2K1*. Two of them (*FAM150B* and *DIS3L-MAP2K1*) were replicated in Asians ($P_{JMA} < 0.05$; refer to the following section). The significant association for JMA test at these loci in Europeans was primarily driven by SNP effects in both lower and higher education strata ($4.40 \times 10^{-8} \leq P \leq 1.35 \times 10^{-6}$, $7.61 \times 10^{-11} \leq P \leq 1.75 \times 10^{-6}$, respectively). SNP x education interaction was not significant ($P_{int} \geq 0.208$). The estimated effect sizes of SNP effects on spherical equivalent were highly similar across education strata.

GEWIS in Asians

The JMA for spherical equivalent in 10,315 individuals from the Asians cohorts identified genome-wide significant association for three genes: *AREG*, *GABRR1* and *PDE10A* ($P_{JMA} < 5.0 \times 10^{-8}$; Table 3 & Figure 2B). SNP x education interaction effects associated with spherical equivalent were observed at all three loci, with genetic effects significantly larger within subjects who had a higher level of education compared with those with a lower education level: *AREG* (rs12511037, β_{int} = -0.89 ± 0.14 D; $P_{int} = 6.87 \times 10^{-11}$), *GABRR1* (rs13215566, β_{int} = -0.56 ± 0.14 D; $P_{int} = 8.48 \times 10^{-5}$) and *PDE10A* (rs12206610, β_{int} = -0.72 ± 0.13 D; $P_{int} = 2.32 \times 10^{-8}$). The genotype and phenotype associations were highly significant in the higher education stratum (main genetic effects $1.97 \times 10^{-10} \leq P \leq 8.16 \times 10^{-8}$) but were considerably weaker in the lower education stratum ($0.008 \leq P \leq 0.243$). There was no evidence of inter-study heterogeneity at index SNPs within *AREG*, *GABRR1* or *PDE10A* (Q test: $P_{het} \geq 0.122$).

Table 1. Characteristics of study participants

Study	N	Study year	Age (SD)	Age range	Male (%)	Spherical Equivalent
Europeans (n=40,036)						
ALIENOR	509	2006 - 2008	79.2 (4.1)	73 - 93	43.2	0.98 (1.98)
ALSPAC	1865	1999 - 2000	45.9 (4.5)	32 - 59	0	-0.76 (2.16)
AREDS	1842	1992	68.1 (4.7)	55 - 81	41.0	0.54 (2.15)
BATS	383	1992 - 2013	24.8 (7.8)	20 - 67	41.3	-0.67 (1.58)
BMES	1896	1992 - 2009	66.8 (8.9)	49 - 94	43.8	0.58 (1.94)
CROATIA-Korcula	807	2007 - 2008	56.2 (13.3)	25 - 94	34.9	-0.13 (1.59)
CROATIA-Split	787	2008 - 2009	51.9 (13.0)	25 - 80	38.6	-1.27 (1.59)
DCCT	1057	1982 - 1993	35.4 (5.8)	25 - 49	54.1	-1.47 (1.80)
EGCUT	904	2002 - 2013	56 (17.0)	25 - 99	38.8	0.33 (3.36)
EPIC	1083	2004 - 2011	68.8 (7.5)	50 - 88	43.8	0.34 (2.27)
ERF	2604	2002 - 2005	48.9 (14.4)	25 - 87	45.0	0.12 (2.03)
FES	2479	1973 - 1975 /1989 - 1991	54.8 (9.3)	28 - 84	55.3	0.27 (2.37)
FITSA	188	2000 - 2001	68.5 (3.3)	63 - 76	0.0	1.44 (2.08)
GHS1	3178	2007 - 2008	55.3 (10.9)	35 - 74	50.4	-0.38 (2.47)
GHS2	1354	2008	54.6 (10.8)	36 - 74	49.6	-0.39 (2.51)
KORA	2326	2004 - 2006	55.1 (11.8)	35 - 84	49.4	-0.26 (2.18)
OGP Talana	456	2002	52.6 (16.3)	25 - 89	57.3	-0.20 (0.24)
ORCADES	1124	2009	56.5 (13.2)	29 - 92	39.1	0.10 (2.07)
RAINE	348	2010 - 2012	20.4 (0.34)	20 - 22	49.1	0.03 (1.29)
RS-I	5702	1991 - 1993	68.7 (8.7)	55 - 99	41.0	0.83 (2.55)
RS-II	2021	2000 - 2002	64.3 (7.9)	55 - 95	46.0	0.48 (2.51)
RS-III	2918	2006 - 2009	56.9 (6.6)	45 - 86	44.0	-0.28 (2.60)
TwinsUK	2154	1998 - 2010	53.8 (11.4)	25 - 84	8.4	-0.96 (2.78)
WESDR	561	1979 - 2007	31.7 (7.0)	25 - 65	50.3	-1.65 (2.07)
YFS	1490	2011	41.9 (5.0)	34 - 49	44.6	-1.09 (2.16)
Asians (n=10,315)						
BES	589	2006 - 2011	62.1 (8.5)	50 - 90	34.0	-0.06 (1.86)
Nagahama	723	2008 - 2010	49.2 (15.2)	30 - 74	33.6	-1.93 (2.46)
SCES I	1710	2009 - 2011	57.5 (7.0)	44 - 84	51.6	-0.72 (2.69)
SCES II	543	2011 - 2012	59.3 (8.9)	46 - 83	51.2	-0.89 (2.74)
SiMES	2256	2004 - 2006	46.8 (10.2)	40 - 80	49.1	-0.03 (1.81)
SINDI	2088	2007 - 2009	55.8 (8.8)	43 - 84	51.5	0.04 (2.07)
SP2-1M	811	1992 - 1998	46.8 (10.2)	25 - 80	62.3	-1.80 (2.84)
SP2-610	854	1992 - 1998	48.4(11.3)	25 - 82	19.6	-1.44 (2.89)
STARS	741	2007 - 2009	38.5 (5.2)	26 - 58	52.4	-2.80 (2.85)

ALIENOR, Antioxydants, Lipids Essentiels, Nutrition et maladies OculaiRes; ALSPAC, Avon Longitudinal Study of Parents and Children; AREDS, Age-Related Eye Disease Study; BATS, Brisbane Adolescent Twins Study; BMES, Blue Mountains Eye Study; DCCT, Diabetes Control and Complications Trial; EGCUT, Estonian Genome Center, University of Tartu; EPIC, EPIC-Norfolk Eye Study; ERF, Erasmus Rucphen Family Study; FES, Framingham Eye Study; FITSA, Finnish Twin Study on Aging; GHS, Gutenberg Health Study; KORA, Cooperative Health Research in the Region of Augsburg; OGP Talana, Ogliastra Genetic Park, Talana study; ORCADES, Orkney Complex Disease Study; RAINE, RAINE Eye Health Study; RS, Rotterdam Study; TwinsUK, Twins UK study; WESDR, Wisconsin Epidemiologic Study of Diabetic Retinopathy; YFS, Young Finns Study; BES, Beijing Eye Study; SCES, Singapore Chinese Eye Study Singapore; SiMES, Singapore Malay Eye Study; SINDI, Singapore Indian Eye Study; SP2, Singapore Prospective Study Program; STARS, Strabismus, Amblyopia, and Refractive Error Study of Preschool Children; SD, standard deviation.; Age is in years; SD, standard deviation.

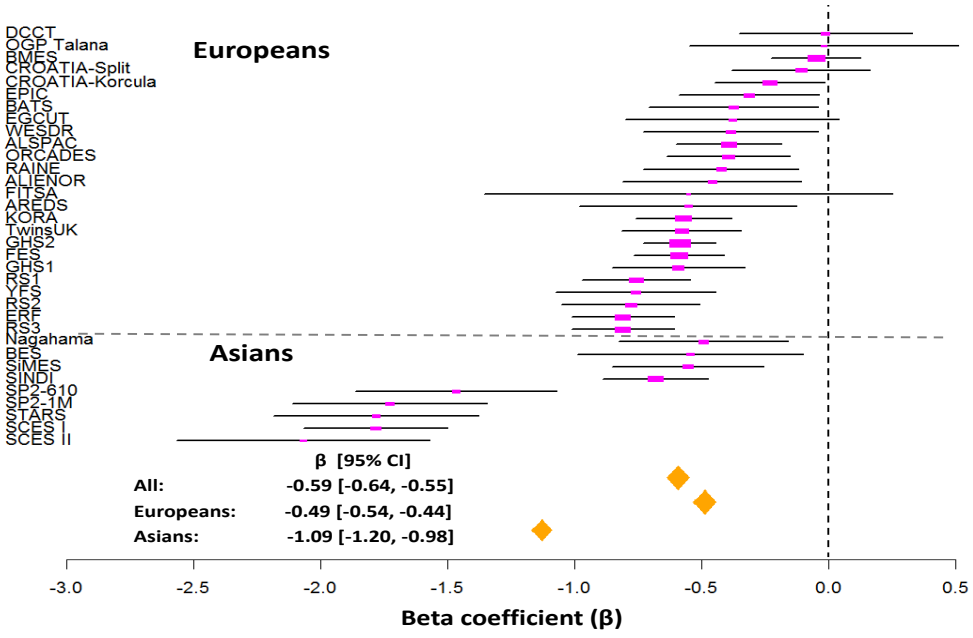

Figure 1. Forest plot of the main effect of education on spherical equivalent across studies
The beta coefficient represents the differences of diopters in refractive error comparing individuals in higher education group versus lower education group**.** The studies are sorted by effect size of education on spherical equivalent within Europeans and Asians studies

GABRR1 and *PDE10A* index SNPs were not associated with spherical equivalent in European samples, for either the JMA test, SNP effect, or SNP education interaction (Table 3). *AREG* SNP rs12511037 was excluded in the meta-analysis of European studies after *quality control filtering (due to MAF < 0.05), hence* a proxy SNP, rs1246413, in LD with rs12511037 (r^2 = 0.67, D' = 1) was tested, whereas insignificant association (P_{JMA} = 0.527; *P* for interaction = 0.176). The meta-regression including study-level characteristics as covariates in the model confirmed the heterogeneity between populations of European and Asian ancestry (*GABRR1*: *P* = 0.006; *PDE10A*: *P* = 0.0419; Supplementary Table 4). For *PDE10A*, besides ethnicity, average spherical equivalent of each study also explained the inter-study heterogeneity for the interaction effects (*P* = 0.025).

We examined whether the underlying assumption of G x E independence held at these three G x E interaction loci. We performed a meta-analysis of logistic regression analysis for education level on *AREG* SNP rs12511037, *GABRR1* SNP rs13215566 and *PDE10A* SNP rs12296610, adjusting for age, gender and population stratification in the Singapore cohorts (n = 9,004). Our analysis did not reveal any significant associations between these loci and education level ($P \geq 0.200$, $P_{het} \geq 0.118$; Supplementary Table 5). Furthermore, the three loci were also not associated with educational attainment in a large meta-analysis of GWAS recently conducted in European cohorts[36]. Thus, our G x E results are unlikely to be biased due to dependence between gene and education[37].

Table 2. Six genetic loci associated with spherical equivalent from the joint meta-analysis in the European populations and combined analysis.

						Europeans (n = 40,306)			Asians (n = 10,315)			All (n = 50,351)	
SNP (Chr:BP)	Gene	Allele	Freq	Subgroup	β	P	P_{het}	β	P	P_{het}	β	P	P_{het}
rs60843830	*FAM150B*	C/G	0.66/0.74	JMA		3.71×10^{-8}	0.086		0.0131	0.980		1.27×10^{-9}	0.395
(2:286756)				Lower educaton	-0.11	4.73×10^{-8}		-0.09	0.010		-0.10	1.65×10^{-9}	
				Higher education	-0.09	1.75×10^{-6}		-0.06	0.509		-0.09	9.83×10^{-7}	
rs10946507	*LINC00340*	A/G	0.47/0.36	JMA		3.07×10^{-8}	0.213		0.433	0.396		2.24×10^{-8}	0.249
(6: 22100367)				Lower educaton	-0.08	7.08×10^{-7}		-0.04	0.313		-0.08	6.13×10^{-7}	
				Higher education	-0.09	1.19×10^{-8}		-0.08	0.450		-0.09	1.20×10^{-8}	
rs8023401	*FBN1*	G/A	0.83/0.85	JMA		1.66×10^{-9}	0.180		0.572	0.979		2.85×10^{-9}	0.495
(15: 48703823)				Lower educaton	-0.15	4.40×10^{-8}		-0.06	0.304		-0.13	8.17×10^{-8}	
				Higher education	-0.16	7.61×10^{-11}		-0.03	0.828		-0.14	2.02×10^{-9}	
rs16949788	*DIS3L-MAP2K1*	T/C	0.91/0.94	JMA		1.34×10^{-8}	0.721		0.0042	0.219		2.19×10^{-8}	0.245
(15: 66590037)				Lower educaton	-0.15	1.35×10^{-6}		0.21	0.103		-0.13	4.88×10^{-6}	
				Higher education	-0.17	1.89×10^{-9}		-0.59	0.014		-0.16	3.90×10^{-9}	
rs10880855	*ARID2*	T/C	0.51/0.43	JMA		7.83×10^{-7}	0.790		0.019	0.779		4.38×10^{-8}	0.867
(12: 46144855)				Lower educaton	-0.09	1.26×10^{-7}		-0.06	0.067		-0.09	8.42×10^{-9}	
				Higher education	-0.07	1.60×10^{-5}		-0.16	0.033		-0.07	3.55×10^{-6}	
rs10853531	*SLC14A2*	G/A	0.70/0.77	JMA		7.82×10^{-6}	0.052		0.0023	0.812		2.54×10^{-8}	0.111
(18: 42824449)				Lower educaton	-0.11	1.27×10^{-6}		-0.15	9.01×10^{-4}		-0.11	3.38×10^{-9}	
				Higher education	-0.08	2.12×10^{-6}		-0.11	0.288		-0.09	7.14×10^{-6}	

JMA, joint meta-analysis on SNP association and SNP × education on spherical equivalent; β, beta coefficient corresponds to the effect in spherical equivalent (diopters) for 1 additional copy of the risk allele in the higher or lower education group. P_{het}, *p*-value for the test of heterogeneity at each SNP; Allele is listed as risk allele/other allele; Freq, allele frequency of the risk allele in Asian/European cohorts.

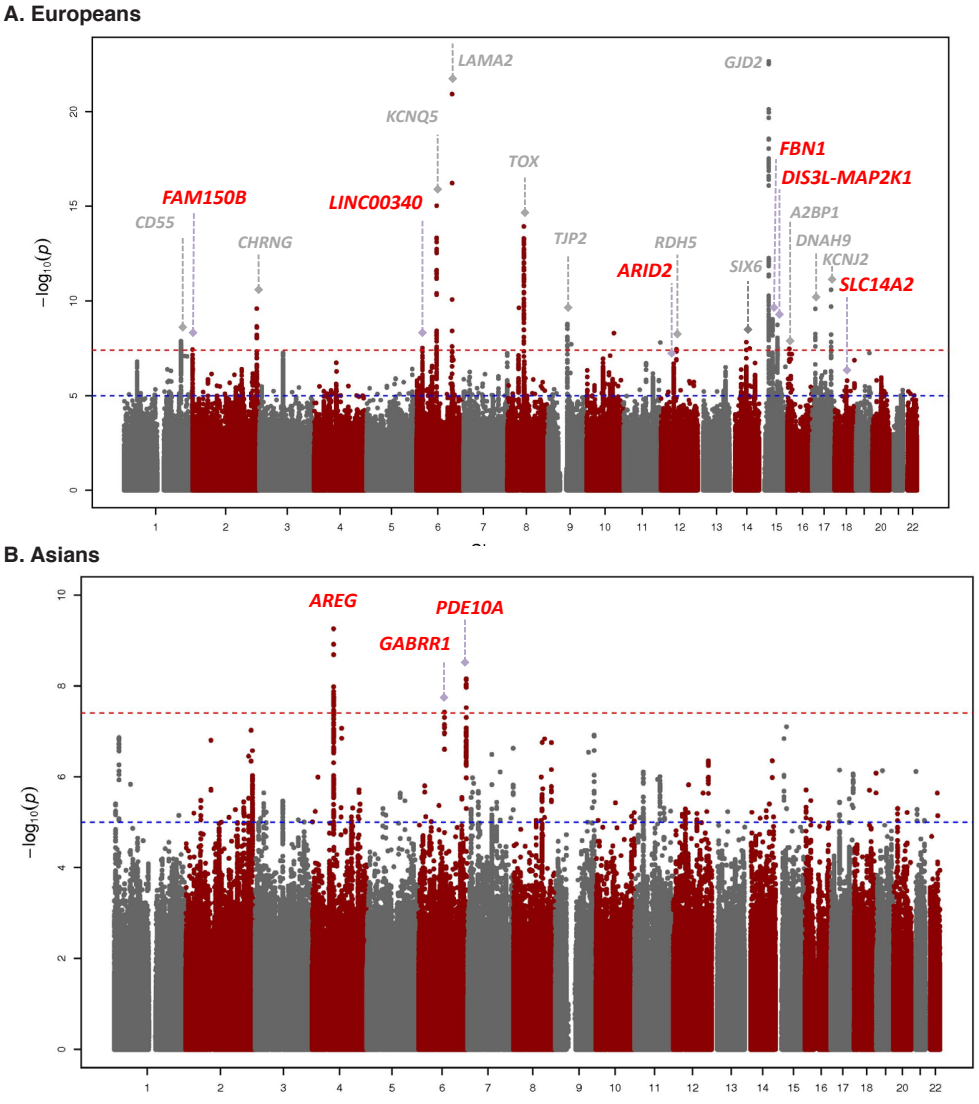

Figure 2. Manhattan plots of $-\log_{10}(P)$ for the Joint meta-analysis on SNP and SNP x education effects on spherical equivalent in A. Europeans and B. Asians

Manhattan plots of $-\log_{10}(P)$ for the Joint meta-analysis on SNP and SNP x education effects on spherical equivalent in A. European Ancestry populations and B. Asian population. The horizontal red line indicates the genome-wide significance level of $p < 5 \times 10^{-8}$. The horizontal blue line indicates the suggestive significance level of $p < 1 \times 10^{-5}$. Novel loci reaching genome-wide significance are labeled in red, and known loci are in grey.

We also evaluated the association for spherical equivalent in Asian cohorts for four loci identified from European populations. Two of them were replicated (*FAM150B*: $P_{JMA} = 0.013$; *DIS3L-MAP2K1:* $P_{JMA} = 0.0042$; Table 2). *DIS3L-MAP2K1* also showed suggestive SNP × education interaction in Asians ($P_{int} = 7.95 \times 10^{-4}$), while this was not significant in Europeans ($P_{int} = 0.208$).

Table 3. Three genetic loci associated with spherical equivalent with a significant SNP x education interaction in Asian populations, and results in European populations

					Asians (n=10,315)			Europeans (n = 40,306)		
SNP (Chr:BP)	Gene	Allele	Freq	Subgroup	β	P	P_{het}	β	P	P_{het}
rs12511037*	AREG	C/T	0.91/0.95	Lower educaiton	0.07	0.243		-0.05	0.323	
(4 :75334864)				Higher education	-0.70	1.97×10^{-10}		-0.03	0.579	
				SNP x education	-0.89	6.87×10^{-11}	0.704	0.02	0.176	0.284
				JMA		5.55×10^{-10}	0.405		0.527	0.186
rs13215566	*GABRR1*	C/G	0.94/0.84	Lower educaiton	-0.13	0.030		-0.03	0.258	
(6: 89918638)				Higher education	-0.68	1.46×10^{-8}		-0.01	0.817	
				SNP x education	-0.56	8.48×10^{-5}	0.134	-0.02	0.459	0.457
				JMA		3.81×10^{-8}	0.122		0.502	0.630
rs12206610	*PDE10A*	C/T	0.90/0.87	Lower educaiton	0.16	0.008		0.01	0.759	
6:166016800				Higher education	-0.59	8.16×10^{-8}		0.01	0.810	
				SNP x education	-0.72	2.32×10^{-8}	0.920	-0.002	0.421	0.111
				JMA		9.21×10^{-9}	0.902		0.954	0.305

JMA, joint meta-analysis on SNP association and SNP x education on spherical equivalent; β (higher education/lower education), beta coefficient corresponds to the effect in spherical equivalent (diopters; D) for 1 additional copy of the risk allele in the higher or lower education group; β (SNP x education), beta coefficient corresponds to the difference in spherical equivalent (D) for 1 additional copy of the risk allele in the higher versus lower education group. β and *p*-value for SNP x education interaction were calculated by the meta-analysis of conducting a 1df Wald test of single interaction parameter. P_{het}, *p*-value for the test of heterogeneity; Allele is listed as risk allele/other allele; FREQ, allele frequency of the risk allele in Asian/European cohorts. *SNP rs12511037 was excluded in met-analysis in European studies because of low MAF (MAF < 0.05). Here we present the results of a proxy SNP rs1246413 (T/G, Freq of risk allele T = 0.95) in LD with rs12511037 (r^2 = 0.67, D' = 1) for European studies.

GEWIS of all cohorts

We subsequently conducted a combined meta-analysis, including both the European and Asian subjects of all 34 studies. This analysis revealed two additional SNPs: *ARID2* (P_{JMA} = 4.38 × 10^{-8}) and *SLC14A2* (P_{JMA} = 2.54 × 10^{-8}). Both loci showed suggestive association with spherical equivalent in European cohorts, while the association was attenuated in Asian cohorts (Table 2). We also detected genome-wide significant associations with spherical equivalent for 17 known loci[8] identified in our previous CREAM GWAS (Supplementary Table 3). The regional plots of the identified novel loci are presented in Supplementary Figure 2.

Gene and education interactions for GWAS known loci

For the previously reported genetic association with spherical equivalent at 39 loci identified from recent two large GWAS[7,8], we evaluated their interactions with education. Two SNP x education interactions were nominally significant (Supplementary Table 6): *TJP2* in Europeans (rs11145488; P_{int} = 6.91 x 10^{-3}) and *SHISA6-DNAH9* in Asians (rs2969180; P_{int} = 4.02 x 10^{-3}). In general, the index SNPs tested at 39 loci had larger SNP x education interaction effect on spherical equivalent in Asians versus Europeans (meta-regression *P* for fold changes < 0.001; Supplementary Figure 3). For 20 SNPs with the same direction of the interaction effect, the magnitudes of interaction effects were 4-fold larger on average in Asians than in Europeans (*P* = 0.003).

Gene and near work interactions for three identified loci

High-level education may reflect an estimator for the greater accumulative effect of near work[38,39]. We thus examined whether there was evidence for SNP x near work interactions associated with spherical equivalent at the three newly-identified loci (*AREG*, *GABRR1* and *PDE10A*) in pediatric cohorts (SCORM, Guangzhou Twins, and ALSPAC; combined n = 5,835; Supplementary Table 7). Tentative support for a SNP x near work interaction was observed for *PDE10A* (rs12206610; P_{int} = 0.032; P_{het} = 0.927), with the stronger genetic effect in children spending more hours on reading, writing or compute use. Weaker support for an interaction was noted at *GABRR1* (rs13215566; P_{int} = 0.109; P_{het} = 0.655), although the direction of meta-analyzed interaction effect was largely consistent across pediatric studies with that observed in adults. We did not observe the interaction at *AREG* (rs12511037: P_{int} = 0.795, P_{het} = 0.062).

Gene expression in human tissues

Using Ocular Tissue Database[34], we examined the expression of the associated genes in 20 normal human donor eyes. The majority of genes identified were expressed in human retina, sclera or retinal pigment epithelium (RPE) (Supplementary Table 8). Among these genes, *GABRR1* had the highest expression in the retina. The PLIER normalized mRNA expression level of *GABRR1* in the retina was 121.7 with an expression value of 21.5 in the sclera, suggesting GABRR1 mRNA is more abundant within the retina. *FAM150B* mRNA was found highly present in the choroid/RPE (expression value of 333.3), while expressed at a much lower level in the retina (29.9). *MAP2K1* was widely expressed in the retina, sclera and choroid with expression values greater than 85.7.

Gene expression of GABRR1 and protein location in mouse ocular tissues

GABA is one of the major inhibitory neurotransmitters in the retina and *GABRR1* encodes the

GABA A receptor[40]. Given that receptors are attractive targets for therapeutic drugs and that GABA has been shown previously to modulate synaptic plasticity in the mammalian nervous system[41], we examined *GABRR1* gene expression in ocular tissues from myopic (spherical equivalent < -5.00 D) mouse eyes compared with age-matched control eyes (Figure 3A)**.** At the mRNA level, *GABRR1* was significantly up-regulated in myopic retina (1.82 fold; $P = 0.012$) when compared to naive controls. By Western blot analysis, retinal GABRR1 protein levels were also up-regulated in myopic versus naive control eyes ($P = 0.025$; Figure 3B).

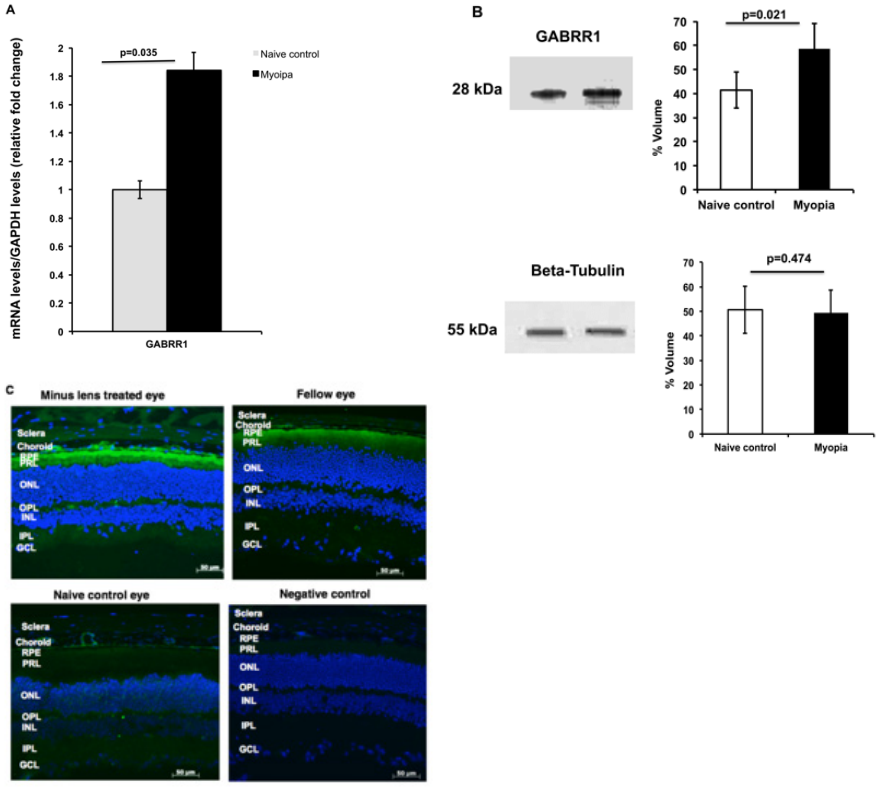

Figure 3. Differential gene expression, protein levels and immunofluorescent labeling of GABRR1 in mice ocular tissues. A. Gene expression levels of GABRR1 in the retina in lens-induced myopic and naïve control eyes. B. Protein levels of GABRR1 in the retina in lens-induced myopic and naïve control eyes. Beta-tubulin was used as a loading control. Western blot analysis of GABRR1 protein eyes showed a pattern of protein expression similar to that of immunohistochemistry analysis. Data represent the mean ± SD; Significance level P≤0.05. C. Immunofluorescent labeling of GABRR1 in lens-induced myopic eyes, contra-lateral controls, and naive controls in mice. The fluorescence intensity labeled of the green color shows the localization of proteins, and blue color indicates the nuclei that were stained with DAPI. The following abbreviations represent the retinal layers: NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRL, photo receptor layer and RPE, retinal pigment epithelium. n = 3 eyes per group and repeated in duplicates. Sections incubated with 4% BSA without primary antibody were utilized as a negative contol.

Additionally, we performed immunofluorescent staining in the mouse myopic retina, choroid and sclera tissue samples to study the localization of GABRR1 protein. GABRR1 protein was present in both the inner and outer retina of all the myopic and contra-lateral fellow eyes and naive controls (Figure 3C). There was higher expression of GABRR1 in the outer retina especially in both photo-receptor layer (PRL) and retinal pigment epithelium (RPE) layers compared to fellow eyes and naive controls.

DISCUSSION

This study represents the most comprehensive genome-wide scan of gene and education interactions to date for refractive error. Here we identified novel genetic loci associated with refractive error by testing the joint contribution of SNP and SNP x education effects in large multi-ethnic populations. Three loci (*AREG*, *GABRR1* and *PDE10A)* showed strong interactions with education in populations of Asian descent, with larger genetic effects within subjects who had a higher level of education compared with those with a lower education level; no interactions achieved statistical significance in Europeans for top JMA associations or known myopic loci. Apart from confirming known associations at 17 previous published loci, we identified six new loci (*FAM150B*, *LINC00340, FBN1, DIS3L-MAP2K1, ARID2* and *SLC14A2*) significantly associated with spherical equivalent using the combined multiracial cohort.

Of the novel loci, *GABRR1* on chromosome 6q15 (53 kb) is an interesting functional candidate suggestive of a role in myopia development. Modulation of synaptic plasticity via GABA-mediated inhibition would be well-placed to alter the "gain" of the visually-guided feedback system controlling refractive development[42]. The lead SNP rs12215566 in *GABRR1*, together with 7 SNPs within the LD block ($r^2 \geq 0.8$), are intronic potentially affecting regulatory motifs (such as zfp128 and gcm1) which may influence transcriptional regulation. As one of the major inhibitory neurotransmitters in the retina, GABA is active in large retinal cells and amacrine cells[14]. Stone and colleagues have reported that antagonists to GABA A, B, and C receptors inhibited form-deprivation myopia in chicks, with greatest effect in the equatorial dimension[43]. GABA receptors also interact with dopamine pathways in the retina[44]. A recent proteomics study determined that levels of GABA transporter-1 (GAT-1) are significantly reduced in myopic murine retina after atropine treatment, implying that GABA signaling is involved in anti-myopic effects of atropine[45]. Therefore, our result in humans is in line with animal experiments, supporting the notion that the GABAergic neurotransmitter signaling pathway in the retina could be a potential player in the progression of myopia.

The rs10889855 on chromosome 6 is an intronic variant within the *ARID2* gene (AT Rich Interactive Domain 2) and about 500kb downstream of *SNAT1 (*Solute Carrier Family 38, Member; Aliases *SLC38A1*). *SNAT1* is a transporter of glutamine, a precursor of GABA[46]. It is also highly expressed in human retina. In our previous meta-analysis in CREAM[8], we identified variants in another glutamate receptor gene *GRIA4* (encoding glutamate receptor, ionotropic); altogether current evidence supports the notion that retinal neurotransmitters GABA and glutamine may be involved in the refractive development.

The strongest association signal for gene and environment interactions was from rs12511037, located 14 kb downstream the *AREG* gene (amphiregulin). AREG is a ligand of the epidermal growth factor receptor (EGFR) promoting the growth of normal epithelial cells, which is critical for cell differentiation and proliferation such as regrowth of the wounded cornea[47]. A link has been found between the muscarinic acetylcholine receptors and the EGFR, as EGFR controls fluid secretion in muscarinic system[48,49].

Another novel association, rs16949788 on chromosome 15, derives from a region that spans *DIS3L and MAP2K1. MAP2K1* encodes mitogen-activated protein kinase 1 which binds to muscarinic receptors during proliferation[50] and inhibits the proliferation of human scleral fibroblasts exposed to all-trans retinoic acid[51]. All-trans retinoic acid is a modulator of ocular growth, inhibiting the proliferation of human scleral fibroblasts[52].

FBN1 (Fibrillin 1) encodes a large extracellular matrix glycoprotein, a member of the fibrillin family. Mutations in *FBN1* cause Marfan's syndrome, a disorder of connective tissue affecting the ocular, skeletal and cardiovascular systems[53]. As a candidate gene for myopia, attempts to study its association with myopia previously produced inconclusive results[54,55], probably due, in part, to underpowered studies with insufficient sample sizes. Using data from a large multi-ethnic population, our results support the role of *FBN1* in myopia development.

The risk alleles of rs12511037 in *AREG*, rs1321556 in *GABRR1*, rs12206610 in *PDE10A* had no or weak influence on myopic shift in the lower education group compared to the higher education group. This suggests that the hereditary predisposition to myopia could be latent for the risk allele carriers, if they are less exposed to the myopiagenic environment associated with high-level education. A lack of strong SNP x near work associations at these loci in pediatric populations leaves open the possibility that environmental risk exposures other than near work might underlie the SNP x education interaction seen in the adult Asian samples.

The genome-wide significant SNPs from the joint meta-analysis approach did not exhibit any interactions with education in Europeans, in contrast to the significant interactive effect among Asians. In particular, the interactions of *AREG*, *GABRR1* and *PDE10A* with education were evident in Asian populations only, but not in Europeans. There are a number of possible reasons. First, the observed heterogeneity may reflect the intense education systems in Asia[1]. The higher education level was associated with myopic shift at an average of a 1.16 D in refraction in Asians, but with only a 0.56 D in Europeans. It is possible that the gene and education interplay may manifest more in such a condition with the strong education effects, as genetic effects are generally modest across the populations. Second, the population distribution of refractive error is more myopic in Asians (-0.60 D versus 0.10 D in Europeans). A high prevalence of myopia is likely to associate with other lifestyle exposures, such as low amount of outdoor activities, which were not accounted for in the current study. Third, education systems varied widely across studies. We chose to divide education levels into two categories but this cut-off may not reflect the same education intensity or true underlying risk for myopia across countries. Misclassification in environment measurements is likely to bias the effect towards the null. Lastly, education in adults may not be an accurate surrogate for cumulative near work activity. The level of education attained may be a crude marker

of reading intensity and computer use during the crucial years prior to the onset of myopia. These factors, accompanying with varying allele frequencies at the associated SNPs, might obscure the power to detect the interaction effects in individuals of European ancestry. Whether such G × E interaction is ancestry-specific warrants further evaluation.

In summary, we identified 9 novel loci associated with refractive error in a large multi-ethnic cohort study by GEWIS approach. Our data provide evidence that specific genetic variants interact with education to influence refractive development, and further support a role for GABA neurotransmitter signaling in myopia development. These findings provide promising candidate genes for follow-up work and may lead to new genetic targets for therapeutic interventions on myopia.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Description of study design, phenotyping and education levels

Study	Method of Measurement	Study design	Higher Education (%)
Europeans			
ALIENOR	Speedy K Luneau, France	Population-based	45.4
ALSPAC	Canon R-50 autorefractor and subjective refraction	Family-based study	38.6
AREDS	Subjective Refraction	Population-based	94.4
BATS	Humphrey-598 Automatic Refractor (USA)	Twins	60.3
BMES	Humphrey autorefractor 530	Population-based	65.5
CROATIA-Korcula	Nidek ARK30 hand-held autorefractometer (Japan)	Family-based	52.7
CROATIA-Split	Nidek ARK30 hand-held autorefractometer	Family-based	83.1
DCCT	Subjective Refraction	Clinic trial	86.2
EGCUT	Autorefraction measurement method; self-reported based on prescription	Population-based	37.2
EPIC	Humphrey Auto-refractor 500	Population-based	62.7
ERF	Topcon RM-A2000 autorefractor	Family-based	29.5
FES	Subjective Refraction	Family-based	53.0
FITSA	Topcon AT (Tokyo, Japan)	Population-based	16.5
GHS1	Humphrey Automated Refractor/ Keratometer (HARK) 599 (Germany)	Population-based	47.1
GHS2	Humphrey Automated Refractor/ Keratometer (HARK) 599 (Germany)	Population-based	49.4
KORA F3	Nikon Retinomax	Population-based	26.5
OGP Talana	Topcon RK-8100 autorefractor	Family-based	16.5
ORCADES	Kowa KW 2000 autorefractometer	Family-based	54.0
RAINE	Nidek ARK-510A	Population-based	73.6
RS-I	Topcon RM-A2000 autorefractor	Population-based	35.3
RS-II	Topcon RM-A2000 autorefractor	Population-based	46.2
RS-III	Topcon RM-A2000 autorefractor	Population-based	53.6
TwinsUK	ARM-10 autorefractor (Takagi Ltd)	Twins study	46.7
WESDR	Subjective Refraction	Clinic trial study	58.4
YFS	Nidek AR-310AR autorefractor	Population-based	85.7
Asians			
BES	Canon RK-5 Auto Ref-Keratometer	Population-based	14.0
Nagahama	NideK ARK-530A	Population-based	75.9
SCES I	Canon RK-5 Auto Ref-Keratometer	Population-based	21.3
SCES II	Canon RK-5 Auto Ref-Keratometer	Population-based	
SIMES	Canon RK-5 Auto Ref-Keratometer	Population-based	6.7
SINDI	Canon RK-5 Auto Ref-Keratometer	Population-based	22.5
SP2-1M	Canon RK-5 Auto Ref-Keratometer	Population-based	45.0
SP2-610	Canon RK-5 Auto Ref-Keratometer	Population-based	37.4
STARS	Canon RK-5 Auto Ref-Keratometer	Population-based	55.7

A higher education group including those who had completed at least higher secondary education, polytechnic, or with ≥ 12 years spent in formal education, except using >12 years of formal education for four cohorts of relatively young European participants (BATS, DCCT, RAINE and WESDER; see Methods).

Supplementary Table S2. Description of genotyping, imputation method and genome control factor lambda (λ_{GC})

Study	Genotyping method	Imputation software	Analysis software	λ_{GC} for JMA
Europeans				
ALIENOR	Illumina HumanHap610-Quad	Minimac	Quicktest	1.049
ALSPAC	Illumina HumanHap660 W-Quad	Minimac	Probabel	1.009
AREDS	Illumina HumanOmni2.5-4v1_B	IMPUTE2	Quicktest	1.056
BATS	Illumina HumanHap610W Quad	Minimac	MIXABLE	1.125
BMES	Illumina HumanHap670 Quad	IMPUTE2	Quicktest	1.026
CROATIA-Korcula	Illumina Human370CNV-Quad	IMPUTE2	MIXABEL	1.054
CROATIA-Split	Illumina Human370CNV-Quad	IMPUTE2	MIxABEL	1.103
DCCT	Illumina Human1M-Omni	IMPUTE2	Quicktest	1.040
EGCUT	Illumina Human OMNIExpress	IMPUTE2	Quicktest	1.021
EPIC	Affymetrix GeneChip Human Mapping 500K	IMPUTE2	Quicktest	1.030
ERF	Illumina 6k, Illumina 318K, Illumina 370K and Affymetrix 250K	Minimac	MIXABEL	1.053
FES	Affymetrix 250K Mapping NspI, 250K Mapping StyI, and HuGeneFocussed 50K	IMPUTE2	MIXABEL	1.012
FITSA	Illumina HumanHap300	IMPUTE2	Quicktest	1.109
GHS1	Affymetrix 6.0	IMPUTE2	Probabel	1.017
GHS2	Affymetrix 6.0	IMPUTE2	Probabel	1.021
KORA	Illumina HumanOmni2.5-4v1_B	IMPUTE2	Quicktest	1.030
OGP Talana	Affymetrix 500k array Chip	IMPUTE2	MIXABEL	1.115
ORCADES	Illumina HumanHap300 & Human370CNV-Quad	IMPUTE2	MIXABEL	1.043
RAINE	Illumina HumanHap610/660 Quad	Minimac	Probabel	1.097
RS-I	Illumina Infinium II & HumanHap550	Minimac	Probabel	1.046
RS-II	Illumina HumanHap550 Duo & HumanHap610-Quad	Minimac	Probabel	1.022
RS-III	Illumina HumanHap610-Quad	Minimac	Probabel	1.024
TwinsUK	Illumina HumanHap300K-Duo & HumanHap610-Quad	IMPUTE2	Quicktest	1.021
WESDR	Illumina Human Omni 1-Quad	IMPUTE2	Quicktest	1.047
YFS	Illumina HumanHap 670k BeadChip	IMPUTE2	Quicktest	1.038
Asians				
BES	Illumina HumanHap610-Quad	Minimac	Quicktest	1.093
Nagahama	HumanHap610KQuad, HumanOmni2.5M, HumanExome	Minimac	Quicktest	1.047
SCES I	Illumina HumanHap610-Quad	Minimac	Quicktest	1.052
SCES II	Illumina HumanHap610-Quad	Minimac	Quicktest	1.072
SIMES	Illumina HumanHap610-Quad	Minimac	Quicktest	1.049
SINDI	Illumina HumanHap610-Quad	Minimac	Quicktest	1.046
SP2-1M	Illumina HumanHap610-Quad	Minimac	Quicktest	1.022
SP2-610	Illumina HumanHap610-Quad	Minimac	Quicktest	1.043
STARS	Illumina HumanHap610-Quad	Minimac	Quicktest	1.022

JMA – Joint meta-analysis

Supplementary Table S3. Previously implicated loci identified from the joint meta-analysis in the combined cohorts

					All (n = 50,351)		Europeans (n=40,036)		Asians (n = 10,315)	
SNP	CHR	POS	Gene	Allele	MAF	JMA P	MAF	JMA P	MAF	JMA P
rs891378	1	207490319	*CD55*	A/G	0.43	3.17E-12	0.43	1.72E-08	0.41	1.30E-04
rs2573210	2	233280565	*CHRNG-ALPPL2*	A/G	0.33	2.79E-10	0.38	2.55E-10	0.12	2.97E-01
rs7744813	6	73643289	*KCNQ5*	A/C	0.43	3.50E-19	0.47	9.43E-16	0.27	2.46E-04
rs12193446*	6	129820038	*LAMA2*	A/G	0.09	1.20E-21	0.09	1.20E-21	0.09	9.33E-01
rs2137277	8	40734662	*ZMAT4*	A/G	0.18	3.99E-09	0.20	1.18E-07	0.10	1.62E-03
rs10089517	8	60178721	*TOX*	A/C	0.36	8.48E-18	0.35	1.17E-14	0.38	6.74E-04
rs11145488	9	71770939	*TJP2*	A/G	0.21	1.56E-09	0.22	1.65E-09	0.09	8.56E-01
rs1649081	10	60292444	*BICC1*	A/G	0.49	2.66E-09	0.48	2.99E-07	0.48	6.60E-03
rs3138142	12	56115585	*RDH5*	T/C	0.20	1.14E-08	0.20	3.57E-08	0.13	2.11E-01
14:54579969:A_AC	14	54579969	*BMP4*	D/R	0.32	9.62E-09	0.34	2.52E-06	0.37	2.19E-03
rs2753462	14	60850703	*SIX6*	C/G	0.29	4.37E-09	0.26	3.88E-08	0.38	3.39E-02
rs524952	15	35005886	*GJD2*	A/T	0.46	1.01E-25	0.47	2.23E-23	0.45	1.31E-04
rs6495367	15	79375347	*RASGRF1*	A/G	0.46	3.89E-08	0.42	5.34E-06	0.39	8.62E-04
rs6500957	16	7462045	*A2BP1*	C/G	0.34	2.86E-09	0.38	3.40E-08	0.12	2.76E-02
rs2908972	17	11407259	*DNAH9*	A/T	0.42	2.73E-12	0.41	2.63E-10	0.46	4.60E-04
rs72483203	17	30463885	*MYO1D-TMEM98*	A/G	0.09	8.81E-09	0.06	0.000482	0.17	9.32E-06
rs929474	17	68724036	*KCNJ2*	A/G	0.42	1.66E-09	0.43	2.58E-11	0.40	3.37E-01

CHR, chromosome; MAF, minor allele frequency. JMA.P, *p*-value for join meta-analysis. Allele is presented as effect allele/other allele.
**LAMA2* rs12193446 was not included in meta-analysis for Asians because of low MAF (MAF < 0.05), a proxy SNP rs9402138 was used.

Supplementary Table S4. Results of meta-regression showing the associations of each study characteristics with the SNP × education interaction effect on spherical equivalent

Study-level characteristics	***GABRR1* (rs13215566)**		***PDE10A* (rs12206610)**	
	Effect	P	Effect	P
Sample size	-	0.662	-	0.636
Average spherical equivalent, D	-	0.205	-	0.025
Proportion of high education group, %	+	0.480	-	0.064
Ethnicity, Asian vs. European	-	0.006	-	0.042
Study year	-	0.409	+	0.397
Study design	+	0.990	-	0.836
Average age ≥ 40 vs. <40, years	+	0.057	-	0.285
Education effect on spherical equivalent, higher vs. lower education	-	0.158	-	0.138

The *P* values were obtained from the meta-regression model, including all the covariates listed above. Study year, the year in the middle of the study period; Study design, independent samples form population-based studies/ clinic trials vs. related samples from family-based studies/twin studies. Meta-regression analysis included all 34 studies in Table 1.

Supplementary Table S5. Associations between three GxE loci and education in Singapore cohorts

SNP	Gene	OR	95% CI of OR		P	P_{het}
rs12511037	*AREG*	0.91	0.79	1.05	0.200	0.224
rs13215566	*GABRR1*	0.98	0.83	1.15	0.769	0.118
rs12206610	*PDE10A*	0.96	0.85	1.08	0.499	0.927

Logistic regression for education on three SNPs was performed in following study (total n = 9,004): SCESI, SCES II, SiMES, SINDI, SP2-1M, SP2-610 and STARS, adjusted for age, gender, and population stratification (SiMES and SINDI). The Odds ratio (OR) was estimated from the meta-analysis of the results from above studies. Education level is defined as 1= higher education, 0= low education.

Supplementary Table S6. Gene and education interaction on spherical equivalent for GWAS identified top loci

						Europeans (n = 40,036)				Asians (n = 10,315)				All (n = 50,351)			
SNP	Chr	Pos	Gene	A1	A2	MAF	β_{int}	s.e	P_{int}	MAF	β_{int}	s.e	P_{int}	MAF	β_{int}	s.e	P_{int}
rs1652333	1	207470460	*CD55*	G	A	0.32	0.002	0.027	0.948	0.40	0.122	0.088	0.165	0.35	-0.012	0.026	0.637
rs4373767	1	219759682	*ZC3H11B*	T	C	0.42	0.010	0.019	0.591	0.38	0.022	0.087	0.802	0.41	0.011	0.018	0.563
rs17412774	2	146773948	*PABPCP2*	A	C	0.45	-0.052	0.024	0.029	0.36	-0.094	0.090	0.294	0.43	-0.054	0.023	0.017
rs17428076	2	172851936	*DLX1*	C	G	0.33	0.032	0.023	0.176	0.16	-0.012	0.155	0.938	0.28	0.031	0.023	0.185
rs1898585	2	178660450	*PDE11A*	T	C	0.28	0.018	0.027	0.515	0.32	-0.064	0.098	0.518	0.29	0.012	0.026	0.649
rs1656404	2	233379941	*PRSS56*	A	G	0.29	0.001	0.035	0.988	0.13	-0.081	0.255	0.751	0.25	-0.001	0.035	0.977
rs1881492	2	233406998	*CHRNG*	T	G	0.23	-0.001	0.035	0.975	0.13	-0.140	0.157	0.370	0.20	-0.008	0.034	0.821
rs14165	3	53847408	*CACNA1D*	G	A	0.33	-0.002	0.022	0.937	0.01	NA	NA	NA	NA	0.000	0.022	0.989
rs13091182	3	141133960	*ZBTB38*	G	A	0.40	-0.034	0.025	0.162	0.00	NA	NA	NA	NA	0.042	0.024	0.087
rs9307551	4	80530671	*LOC100506035*	A	C	0.25	-0.008	0.037	0.840	0.47	-0.030	0.086	0.729	0.31	-0.011	0.034	0.747
rs5022942	4	81959966	*BMP3*	A	G	0.25	-0.013	0.029	0.646	0.40	0.074	0.092	0.423	0.29	-0.006	0.028	0.843
rs7744813	6	73643289	*KCNQ5*	A	C	0.42	-0.027	0.028	0.336	0.30	-0.032	0.098	0.742	0.38	-0.027	0.027	0.310
rs9492338	6	129842538	*LAMA2*	C	G	0.23	-0.041	0.030	0.173	0.09	-0.007	0.161	0.967	0.19	-0.040	0.030	0.178
rs7829127	8	40726394	*ZMAT4*	A	G	0.31	-0.063	0.035	0.068	0.10	-0.140	0.141	0.322	0.25	-0.067	0.034	0.045
rs7837791	8	60179086	*TOX*	G	T	0.49	-0.018	0.023	0.426	0.43	0.065	0.089	0.466	0.47	0.013	0.022	0.558
rs4237036	8	61701057	*CHD7*	T	C	0.35	0.005	0.023	0.842	0.38	0.074	0.105	0.484	0.36	0.008	0.022	0.732
rs11145488	9	71770939	*TJP2*	A	G	0.31	-0.104	0.038	0.007	0.09	0.006	0.274	0.983	0.24	-0.101	0.038	0.008
rs7042950	9	77149837	*RORB*	G	A	0.31	-0.048	0.033	0.139	0.31	-0.021	0.098	0.829	0.31	0.046	0.031	0.141
rs7084402	10	60265404	*BICC1*	G	A	0.48	-0.029	0.025	0.238	0.47	-0.023	0.087	0.789	0.48	0.029	0.024	0.227
rs6480859	10	79081948	*KCNMA1*	T	C	0.42	0.022	0.020	0.291	0.15	0.018	0.120	0.883	0.34	0.022	0.020	0.287
rs745480	10	85986554	*RGR*	G	C	0.49	0.019	0.019	0.312	0.41	-0.037	0.087	0.668	0.47	-0.017	0.019	0.370
rs10882165	10	94924324	*CYP26A1*	T	A	0.44	0.002	0.020	0.914	0.24	0.116	0.171	0.498	0.38	-0.004	0.020	0.854
rs1381566	11	40149607	*LRRC4C*	G	T	0.30	-0.004	0.038	0.917	0.33	-0.055	0.110	0.613	0.31	0.009	0.036	0.792
rs2155413	11	84634790	*DLG2*	A	C	0.47	-0.001	0.024	0.960	0.26	0.104	0.104	0.318	0.41	0.004	0.023	0.859
rs11601239	11	105556598	*GRIA4*	C	G	0.47	0.015	0.017	0.384	0.42	-0.019	0.087	0.832	0.46	0.014	0.017	0.417
rs3138142	12	56115585	*RDH5*	C	T	0.33	-0.024	0.034	0.491	0.05	0.263	0.297	0.376	0.28	0.020	0.034	0.560
rs12229663	12	71249996	*PTPRR*	A	G	0.32	-0.011	0.023	0.639	0.38	-0.045	0.091	0.619	0.34	-0.013	0.022	0.566
rs8000973	13	100691367	*ZIC2*	C	T	0.48	0.014	0.027	0.606	0.27	0.000	0.103	0.997	0.42	-0.013	0.026	0.617
rs2184971	13	100818092	*PCCA*	A	G	0.46	-0.013	0.024	0.598	0.28	0.138	0.098	0.159	0.41	-0.004	0.024	0.863
rs66913363	14	54413001	*BMP4*	G	C	0.49	-0.015	0.020	0.461	0.34	0.007	0.105	0.945	0.44	0.014	0.020	0.477
rs1254319	14	60903757	*SIX6*	A	G	0.36	0.000	0.026	0.987	0.36	0.051	0.090	0.569	0.36	0.004	0.025	0.863
rs524952	15	35005886	*GJD2*	A	T	0.47	0.006	0.023	0.802	0.44	-0.104	0.087	0.231	0.46	-0.002	0.022	0.947
rs4778879	15	79372875	*RASGRF1*	G	A	0.45	-0.007	0.026	0.779	0.42	-0.100	0.090	0.264	0.44	0.015	0.025	0.560
rs17648524	16	7459683	*A2BP1*	C	G	0.40	0.005	0.023	0.833	0.20	-0.161	0.147	0.273	0.34	0.001	0.023	0.970
rs2969180	17	11407901	*SHISA6*	A	G	0.39	-0.012	0.028	0.667	0.46	-0.257	0.089	0.004	0.41	-0.034	0.027	0.203
rs17183295	17	31078272	*MYO1D*	T	C	0.29	-0.012	0.028	0.672	0.01	NA	NA	NA	NA	-0.011	0.028	0.704
rs4793501	17	68718734	*KCNJ2*	T	C	0.43	-0.003	0.023	0.898	0.42	0.164	0.089	0.064	0.43	0.008	0.023	0.731
rs12971120	18	72174023	*CNDP2*	A	G	0.32	0.002	0.024	0.942	0.29	-0.038	0.094	0.684	0.31	-0.001	0.023	0.976
rs235770	20	6761765	*BMP2*	T	C	0.39	-0.009	0.022	0.696	0.31	0.073	0.103	0.480	0.37	-0.005	0.022	0.815

β_{int}, Beta regression coefficient for SNP and education interaction on spherical equivalent. P_{int}, *p-value* for interaction between SNP and education on spherical equivalent. A1-risk allele; A2- other allele; Genome NCBI build 37. NA; not applicable due to low MAFs in Asian studies

Supplementary Table S7. Meta-analysis of gene and near work interaction for spherical equivalent in pediatric cohorts on three index SNPs

SNP	Gene	A1	A2	Effect	s.e.	P_{int}	Direction	P_{het}
rs12511037	*AREG*	C	T	0.045	0.173	0.795	+-+	0.062
rs13215566	*GABRR1*	C	G	-0.088	0.066	0.309	---	0.655
rs12206610	*PDE10A*	C	T	-0.189	0.088	0.032	---	0.658

Meta-analysis of SNP x near work was performed in Chinese children from SCORM (n = 988), Guangzhou twins (n = 1,055) and European children in ALSAPC[3; 4] (n = 3,792). Near work is a binary variable, defined as
0 = low and 1= high, relative to the median number of hours per week spent reading, writing or computer use. Only near work activity outside of the regular school day was included. SCORM: Singapore Cohort study Of the Risk factors for *Myopia*; ALSPAC: Avon Longitudinal Study of Parents and Children. Genotyping GWAS were available from three cohorts.

Supplementary Table S8. Gene expression of identified loci in human ocular tissues

Gene	Retina	Sclera	Choroid / RPE
FAM150B	29.94	62.13	333.33
PRL	43.48	24.74	43.64
FBN1	12.88	75.26	47.08
MAP2K1	85.72	91.26	183.61
DIS3L	43.20	32.95	42.16
SLC14A2	29.96	34.87	33.69
AREG	21.31	26.04	29.64
GABRR1	121.66	21.48	31.43
PDE10A	28.19	18.87	21.46

Expression data was obtained from Ocular Tissue Database[47]. The Affymetrix GeneChip Human Exon 1.0 ST (HuEx 1.0) microarrays were used to assess gene expression. Normalization of gene expression was done at both the probe set and metaprobe set level using the Probe Logarithmic Intensity Error (PLIER) method with GC-background correction. The PLIER normalized level of gene expression was presented in the table.

Supplementary Table S9. Regulatory function for the index SNP and SNPs in linkage disequilibrium (r2 ≥ 0.8)

Query SNP: rs12511037 and variants with r^2 >= 0.8

variant	Promoter histone marks	Enhancer histone marks	DNAse	Proteins bound	Motifs changed	GENCODE genes	dbSNP func annot
rs12511037					CEBPG	14kb 3' of AREG	
rs2201455		HMEC, NHEK			4 altered motifs	37kb 3' of AREG	
rs2643009					Cdx2,Pdx1	37kb 3' of AREG	
rs1971299					NRSF	41kb 3' of AC142293.3	
rs1494885					Foxa	40kb 3' of AC142293.3	
rs1817910					Ets,Gm397	37kb 3' of AC142293.3	
rs1389962					21 altered motifs	32kb 3' of AC142293.3	
rs78293098			FibroP		8 altered motifs	24kb 3' of AC142293.3	
rs4694198		HMEC, Huvec, NHEK			Dlx3,Sox	5kb 3' of AC142293.3	

Query SNP: rs13215566 and variants with r^2 >= 0.8

variant	Promoter histone marks	Enhancer histone marks	DNAse	Proteins bound	Motifs changed	GENCODE genes	dbSNP func annot
rs12374613		H1			6 altered motifs	GABRR1	intronic
rs35953049			Medullo		4 altered motifs	GABRR1	intronic
rs13196063					4 altered motifs	GABRR1	intronic
rs13196423					13 altered motifs	GABRR1	intronic
rs35124757					Mef2,TATA	GABRR1	intronic
rs13215029			HRPEpiC, SK-N-MC		5 altered motifs	GABRR1	intronic
rs13201083					CTCF,NERF1a, RFX5	GABRR1	intronic
rs13215566					Gcm1,Pax-6, Zfp128	GABRR1	intronic
rs35007480			Osteobl	FOXA1, GATA3	Foxd3,HDAC2, Pou3f2	GABRR1	intronic

Query SNP: rs12206610 and variants with r^2 >= 0.8

variant	Promoter histone marks	Enhancer histone marks	DNAse	Proteins bound	Motifs changed	GENCODE genes	dbSNP func annot
rs12216245					DMRT3	PDE10A	intronic
rs62426699						PDE10A	intronic
rs62426700					Evi-1,Gfi1	PDE10A	intronic
rs12214904					TLX1::NFIC	PDE10A	intronic
rs12206610					Foxd3,Sox, Zfp105	PDE10A	intronic
rs12215013					Foxa	PDE10A	intronic
rs12192968					LUN-1	PDE10A	intronic
rs12206770					ERalpha-a,Spz1,TCF12	PDE10A	intronic

rs62426701				Foxp3	PDE10A	intronic
rs62426702				ATF3,Pou2f2, TCF11::MafG	PDE10A	intronic
rs76154906					PDE10A	intronic
rs76510607				Dobox4,SIX5	PDE10A	intronic
rs76914213				Mrg1::Hoxa9	PDE10A	intronic
rs11751207				5 altered motifs	PDE10A	intronic
rs199547339				12 altered motifs	PDE10A	intronic
rs78291302					PDE10A	intronic
rs11751728				4 altered motifs	PDE10A	intronic
rs12210339			HMVEC-LLy		PDE10A	intronic
rs12190475			4 cell types	4 altered motifs	PDE10A	intronic
rs12191985			4 cell types	GR,HNF4	PDE10A	intronic
rs12210393			4 cell types	4 altered motifs	PDE10A	intronic
rs12192105			Jurkat	EWSR1-FLI1,TATA,p300	PDE10A	intronic
rs12210507			Jurkat,RPTEC	DMRT7,YY1	PDE10A	intronic
rs12212289		HSMM		AP-1,Mef2	PDE10A	intronic
rs12198402		HSMM	HCPEpiC	Pou3f3	PDE10A	intronic
rs12198517		HSMM	Jurkat	4 altered motifs	PDE10A	intronic
rs11752590				PLZF	PDE10A	intronic
rs12195874				NRSF	PDE10A	intronic
rs12195883				Hltf,Pou1f1,Pou5f1	PDE10A	intronic
rs828571				9 altered motifs	PDE10A	intronic
rs12213759				E2F,TATA,YY1	PDE10A	intronic
rs12209263				Pax-4,SIX5,Znf143	PDE10A	intronic
rs12204986				PTF1-beta	PDE10A	intronic
rs12196646				7 altered motifs	PDE10A	intronic
rs12196655				7 altered motifs	PDE10A	intronic
rs12206474				7 altered motifs	PDE10A	intronic
rs12206582			10 cell types	5 altered motifs	PDE10A	intronic
rs12198136			10 cell types	6 altered motifs	PDE10A	intronic
rs12211245			5 cell types	5 altered motifs	PDE10A	intronic
rs142625747					PDE10A	intronic
rs12205255				HNF4,Sox	PDE10A	intronic
rs12200612				5 altered motifs	PDE10A	intronic
rs62424870					PDE10A	intronic
rs60457032				6 altered motifs	PDE10A	intronic
rs57345708			HMVEC-dBl-Neo	NF-I	PDE10A	intronic
rs12212598				PLZF	PDE10A	intronic
rs12206551			Osteobl	Ik-1,Spz1,Zec	PDE10A	intronic
rs12208043					PDE10A	intronic

Query SNP: rs60843830 and variants with r^2 >= 0.8

variant	*Promoter histone marks*	*Enhancer histone marks*	*DNAse*	*Proteins bound*	*Motifs changed*	*GENCODE genes*	*dbSNP func annot*
rs62114494					37 altered motifs	6.4kb 3' of SH3YL1	
rs2126129					7 altered motifs	5.2kb 3' of SH3YL1	
rs62114497		NHEK			MIZF	2.9kb 3' of SH3YL1	

rs6709534					5 altered motifs	395bp 3' of SH3YL1	
rs56350804			PanIsletD		9 altered motifs	169bp 3' of SH3YL1	
rs200781940			PanIsletD		10 altered motifs	167bp 3' of SH3YL1	
rs9213					Ets,SIX5	SH3YL1	3'-UTR
rs3828165					5 altered motifs	SH3YL1	intronic
rs60484953					6 altered motifs	SH3YL1	intronic
rs3791224					4 altered motifs	SH3YL1	intronic
rs3791223					Pou5f1,RBP-Jkappa	SH3YL1	intronic
rs2290911					BRCA1,NF-I,RFX5	SH3YL1	synonymous
rs3791221						SH3YL1	intronic
rs3791220					4 altered motifs	SH3YL1	intronic
rs17713396						SH3YL1	intronic
rs57542652			Th2		Foxp3,NF-AT1	SH3YL1	intronic
rs7601944					11 altered motifs	SH3YL1	intronic
rs2306060					PRDM1	SH3YL1	intronic
rs62114501			Hepatocytes		4 altered motifs	SH3YL1	intronic
rs3838489					19 altered motifs	SH3YL1	intronic
rs6710091					4 altered motifs	SH3YL1	intronic
rs4497901					GR	SH3YL1	intronic
rs17713568					6 altered motifs	SH3YL1	intronic
rs62114505					Evi-1	SH3YL1	intronic
rs55753056					4 altered motifs	SH3YL1	intronic
rs17713729		HepG2			DMRT3,DMRT4, DMRT5	SH3YL1	intronic
rs17713879		K562			Nkx2	SH3YL1	intronic
rs62114538					Foxp1	SH3YL1	intronic
rs55936726		HepG2		SETDB1		SH3YL1	intronic
rs36216559	NHEK	HepG2, HMEC	6 cell types	HEY1,POL2	GR,Nkx2	SH3YL1	intronic
rs7595075	8 cell types	HepG2	19 cell types	5 bound proteins	AP-2,BDP1	SH3YL1	5'-UTR
rs7584915	8 cell types	HepG2	H1-hESC, 8988T,Th2	ZEB1,POL2	BDP1,ELF1, HNF4	ACP1	
rs58461606	K562, GM12878	HepG2, NHLF, HMEC			GR	ACP1	intronic
rs56321614	K562	HepG2, GM12878	CMK,HL-60		Irf,TAL1	ACP1	intronic
rs55946380	K562, GM12878	HepG2	HL-60		Cdx,p300	ACP1	intronic
rs62114544	GM12878				Znf143	ACP1	intronic
rs59937473						ACP1	intronic
rs11553746			Th1,Fibrobl, HL-60		4 altered motifs	ACP1	missense
rs62114548			6 cell types	CTCF,RAD21, AP2GAMMA	AP-2,ZEB1	ACP1	intronic
rs7605824					E2F	FAM150B	intronic
rs7566279			Fibrobl			FAM150B	intronic
rs56167434			Fibrobl	POL2	NRSF,Sin3Ak-20,p53	FAM150B	intronic
rs60149603		H1, NHLF			4 altered motifs	FAM150B	intronic
rs17714252		H1, NHLF				FAM150B	intronic

rs60843830	H1		WI-38		ERalpha-a,Pbx-1	FAM150B	intronic
rs79154857					CTCFL,TAL1	AC079779.4	

Query SNP: rs10946507 and variants with r^2 >= 0.8

variant	*Promoter histone marks*	*Enhancer histone marks*	*DNAse*	*Proteins bound*	*Motifs changed*	*GENCODE genes*	*dbSNP func annot*
rs10946507			7 cell types		GCNF,NF-I, Pou1f1	LINC00340	intronic
rs5874850					Foxp1,HMG-IY, Zfp105	LINC00340	intronic
rs964461					BCL	LINC00340	intronic
rs12216030		GM12878, NHLF		4 bound proteins	PPAR	LINC00340	intronic

Query SNP: rs8023401 and variants with r^2 >= 0.8

variant	*Promoter histone marks*	*Enhancer histone marks*	*DNAse*	*Proteins bound*	*Motifs changed*	*GENCODE genes*	*dbSNP func annot*
rs201102733					6 altered motifs	9.9kb 3' of FBN1	
rs8032307		HSMM, NHLF	15 cell types		CDP,HNF1	7.9kb 3' of FBN1	
rs8032308		HSMM, NHLF	14 cell types		CDP,HNF1	7.9kb 3' of FBN1	
rs12592059					CEBPG,E2F, Pou3f2	4.1kb 3' of FBN1	
rs2899417					GATA,Rad21	399bp 3' of FBN1	
rs13598						FBN1	3'-UTR
rs8023401						FBN1	intronic
rs13379564					5 altered motifs	FBN1	intronic
rs1820488		H1				FBN1	intronic
rs8028152						FBN1	intronic
rs9920665		HMEC, NHEK			GR,Maf	FBN1	intronic
rs2042746			15 cell types		Nkx2	FBN1	intronic
rs8029557		NHLF, H1			Zic	FBN1	intronic
rs2278185			5 cell types		6 altered motifs	FBN1	intronic
rs201882828					HNF1,Mef2	FBN1	intronic
rs2466791		Huvec			GR,Sox	FBN1	intronic
rs2017765					STAT,Znf143	FBN1	intronic
rs34539187						FBN1	intronic
rs11855195					4 altered motifs	FBN1	intronic
rs75227249					Mef2,ZBTB33	FBN1	intronic
rs12907167					Pou2f2,Pou3f2	FBN1	intronic
rs17361098					4 altered motifs	FBN1	intronic
rs34215103					Pou2f2	FBN1	intronic
rs16960982					4 altered motifs	FBN1	intronic
rs12917479					8 altered motifs	FBN1	intronic
rs71467652		H1			Hoxa5	FBN1	intronic
rs34070783					5 altered motifs	FBN1	intronic
rs16960997		NHLF			5 altered motifs	FBN1	intronic
rs17458846					HNF1	FBN1	intronic
rs12915497					Ets,TLX1::NFIC, YY1	FBN1	intronic
rs12915240					TLX1::NFIC,YY1	FBN1	intronic

rs12901992					FBN1	intronic
rs12907671				7 altered motifs	FBN1	intronic
rs34837775		HSMM, NHLF		Nkx2	FBN1	intronic
rs12914007		GM12878		CEBPB,p300	FBN1	intronic
rs35464791				4 altered motifs	FBN1	intronic
rs35716640			CTCF	AIRE,Pax-4, Sin3Ak-20	FBN1	intronic
rs11854914		Huvec			FBN1	intronic
rs12909189				6 altered motifs	FBN1	intronic
rs34054358				Hoxa5,Sin3Ak-20	FBN1	intronic
rs17460049					FBN1	intronic
rs17362691			CTCF	GATA,ZEB1, Zfp410	FBN1	intronic
rs2279237					FBN1	intronic
rs1871483				HNF4	FBN1	intronic

Query SNP: rs16949788 and variants with r^2 >= 0.8

variant	*Promoter histone marks*	*Enhancer histone marks*	*DNAse*	*Proteins bound*	*Motifs changed*	*GENCODE genes*	*dbSNP func annot*
rs16949788					18 altered motifs	DIS3L	intronic
rs76878359					Maf,NRSF,PLZF	DIS3L	intronic
rs16949793			HeLa-S3		Mrg,Nanog,Sox	DIS3L	intronic
rs9806600			H7-hESC			DIS3L	intronic
rs142910616					Mef2,ZBTB33	DIS3L	intronic
rs8035939					Pbx3	DIS3L	intronic
rs28723485					EBF,Ik-1	DIS3L	intronic
rs11071885					Ets,Irf	DIS3L	synonymous
rs62625678					17 altered motifs	487bp 3' of TIPIN	
rs62625675					Foxo,HDAC2,YY1	TIPIN	3'-UTR
rs62627323					GR,Smad	TIPIN	intronic
rs9806474			Hepatocytes		AP-1,GATA,Smad4	TIPIN	intronic
rs12443313		HepG2			BCL,CHD2,E2F	TIPIN	
rs8042604						TIPIN	
rs12323975					Nanog	TIPIN	
rs16949849		6 cell types	Hepatocytes, Osteobl		6 altered motifs	MAP2K1	intronic
rs80298548					4 altered motifs	MAP2K1	intronic

Query SNP: rs10880855 and variants with r^2 >= 0.8

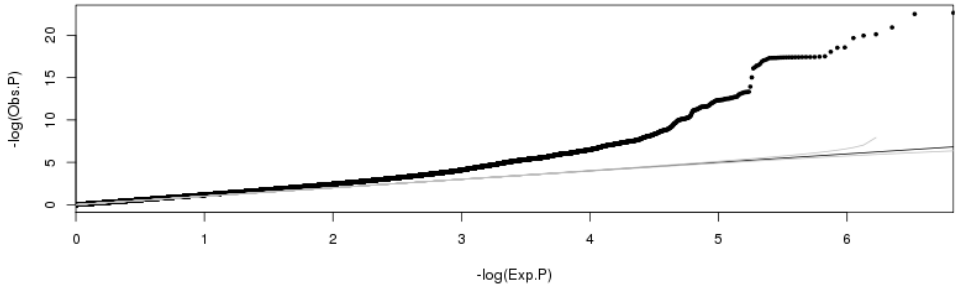
variant	*Promoter histone marks*	*Enhancer histone marks*	*DNAse*	*Proteins bound*	*Motifs changed*	*GENCODE genes*	*dbSNP func annot*
rs67133230	9 cell types		61 cell types	5 bound proteins	4 altered motifs	ARID2	intronic
rs7138997					4 altered motifs	ARID2	intronic
rs2193749					DMRT1	ARID2	intronic
rs10880855					10 altered motifs	ARID2	intronic
rs1468993						ARID2	intronic
rs12320533					4 altered motifs	ARID2	intronic
rs12319077						ARID2	intronic

rs11183201				13 altered motifs	ARID2	intronic
rs201967811				5 altered motifs	ARID2	intronic
rs142543635				6 altered motifs	ARID2	intronic
rs10748432				Cdx,STAT	ARID2	intronic
rs201070908				5 altered motifs	ARID2	intronic
rs79637844				4 altered motifs	ARID2	intronic
rs7132422				10 altered motifs	ARID2	intronic
rs7955891				5 altered motifs	ARID2	intronic
rs2408435					ARID2	intronic
rs35671385				18 altered motifs	ARID2	intronic
rs201994368				5 altered motifs	ARID2	intronic
rs72215781					ARID2	intronic
rs10880859				34 altered motifs	ARID2	intronic
rs1863127				4 altered motifs	ARID2	intronic
rs6582574					ARID2	intronic
rs10880860				7 altered motifs	ARID2	intronic
rs2059404					ARID2	intronic
rs141510569				5 altered motifs	ARID2	intronic
rs7976870				Ik-2,Irf,TCF4	ARID2	intronic
rs247930					ARID2	intronic
rs35117			HMVEC-LBl	Irf,Pax-4,STAT	ARID2	intronic
rs35115				KAP1	ARID2	intronic

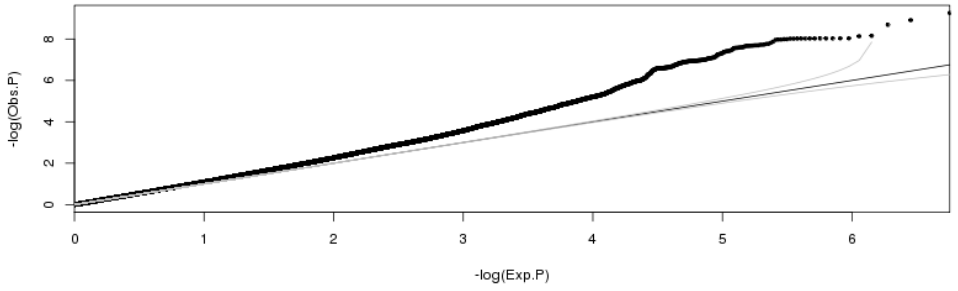
Query SNP: rs10853531 and variants with r^2 >= 0.8

variant	*Promoter histone marks*	*Enhancer histone marks*	*DNAse*	*Proteins bound*	*Motifs changed*	*GENCODE genes*	*dbSNP func annot*
rs11659892					DMRT5	175kb 3' of SETBP1	intronic
rs11659914					GATA,HDAC2	175kb 3' of SETBP1	intronic
rs16978310		HSMM, NHLF, NHEK	6 cell types		YY1	175kb 3' of SETBP1	intronic
rs7235910		NHEK, HMEC	BJ	GATA3	Egr-1,Hbp1	176kb 3' of SETBP1	intronic
rs10853531					CACD,NRSF,Pax-4	176kb 3' of SETBP1	intronic

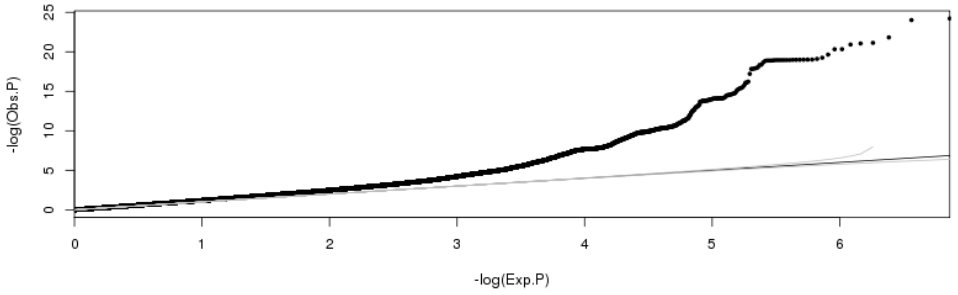
A. Europeans



B. Asians

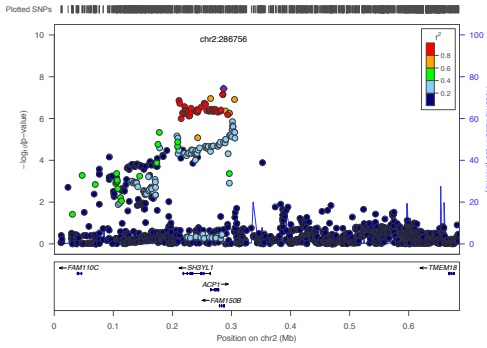


C. All

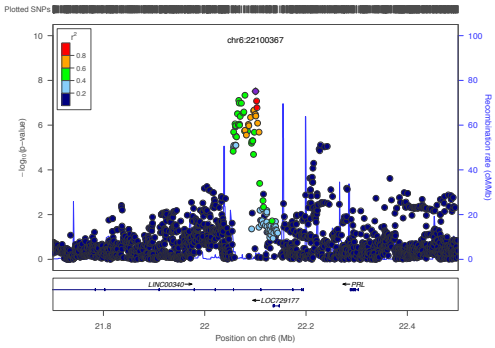


Supplementary Figure S1. Quantile-quantile (QQ) plots for the join meta-analysis in Europeans, Asians and all cohorts

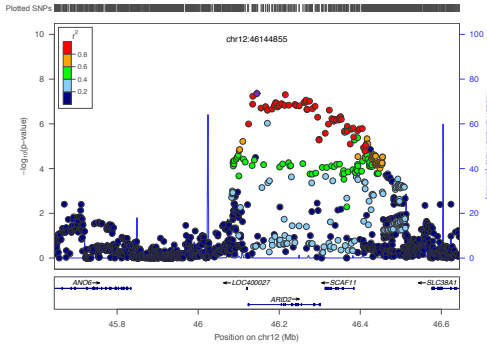
A. rs60843830



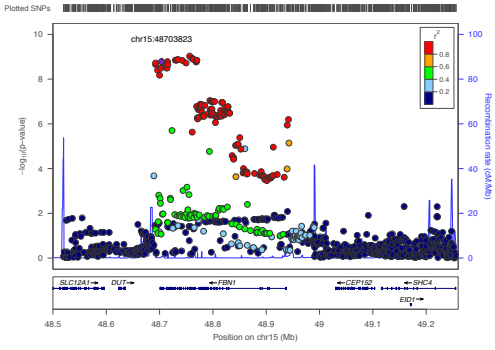
B. rs10946507



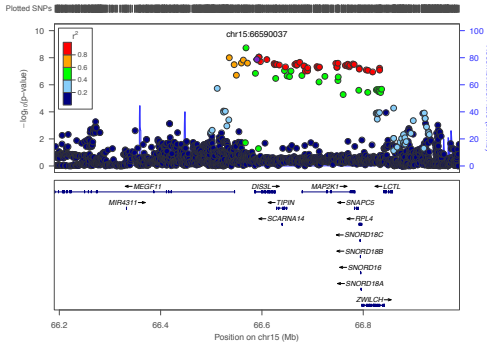
C. rs10880855



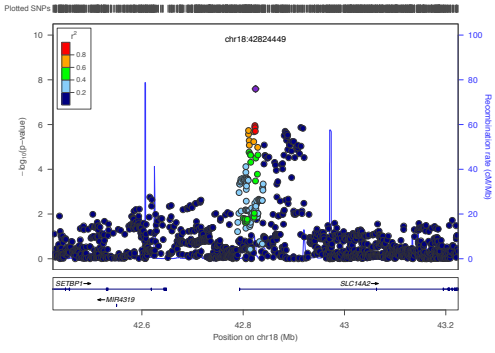
D. rs8023401



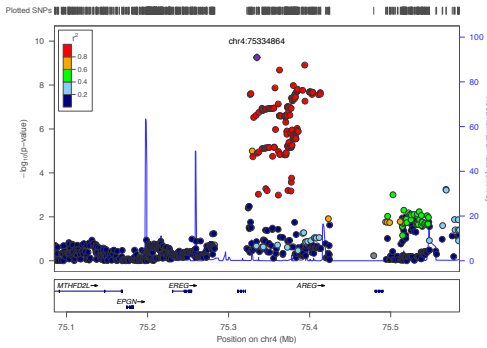
E. rs16949788



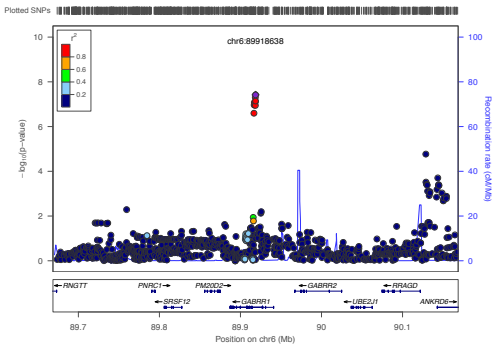
F. rs10853531



G. rs12511037



H. rs13215566



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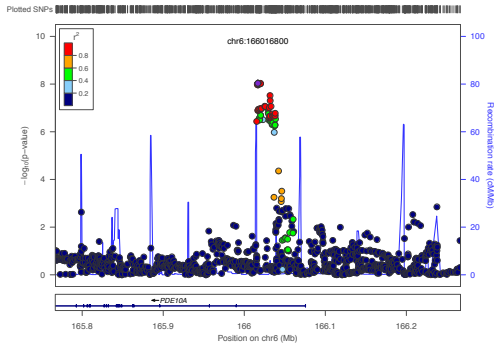
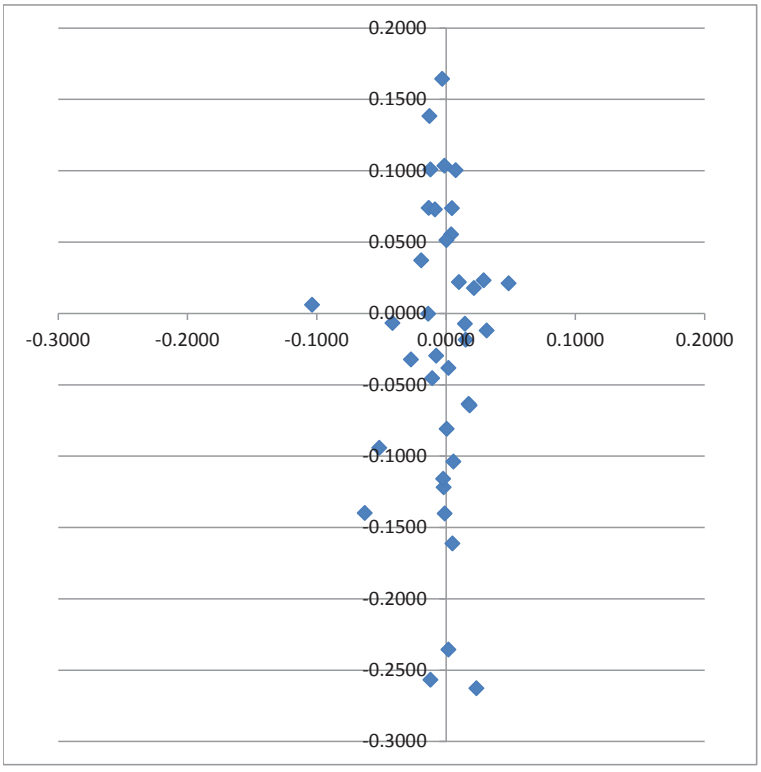


Supplementary Figure S2. Regional association plots of the loci associated with spherical equivalent for the join meta-analysis. A-F. regional plots for all studies. G-I. regional plots for Asian studies.



Supplementary Figure S3. Scatter plot of effects of SNP x education interaction on 39 known GWAS loci

The Meta-regression p values were obtained from the meta-regression with the outcome is the fold-changes of the interaction beta coefficients from Asians versus Europeans. Beta coefficient corresponds to the effect of 1 additional copy of the risk allele on spherical equivalent in the high vs low educational setting.

5

Development of refractive errors – what can we learn from retinal dystrophies?

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Manuscript submitted.

ABSTRACT

Purpose

Myopia is hypothesized to be caused by a retina-to-sclera signaling cascade. It is unknown which retinal cells are involved in this process. We aimed to get hints of cell types and functions involved by studying refractive errors in Mendelian inherited retinal dystrophies (RD).

Methods

We included patients with Mendelian RD from three ophthalmogenetic centers (n=302) in the Netherlands. Patients were categorized by primary affected cell type: retinal pigment epithelium-related macular dystrophies (RPE, n=77), cone-related dystrophies (CD, n=76), rod-related dystrophies, such as retinitis pigmentosa (RP, n=104), and bipolar cell dysfunction related dystrophies (BPD, n=45). Refractive error was measured with automated refraction, and analyzed as spherical equivalent (SE). Frequency distributions and mean SE of each diagnosis and causal gene were calculated, and risks of several degrees of myopia and hyperopia versus emmetropia was evaluated using logistic regression analyses, adjusting for age and sex. Reference for risk analyses were the population-based estimates from the Rotterdam Study III and ERF Study (n=5,550).

Results

Participants with BPD (mean SE -6.86 D [SD 6.38]) had the highest degree of refractive error, followed by CD (mean SE -3.10 D [SD 4.49]), RD (mean SE -2.27 D [SD 4.65]), and RPE related macular dystrophies (mean SE -0.10 D[SD 3.09]). Patients with mutations in the *RPGR* gene (causing X-linked retinitis pigmentosa; mean SE -7.63 D (SD)) and *CACNA1F* gene (causing congenital stationary night blindness; mean SE -5.33 D (SD)) presented with the highest degree of myopia. Persons with bipolar cell dysfunctions had the highest risk of refractive error (OR high myopia 239.7 and OR mild hyperopia 263.2, both $P<0.0001$); followed by cone related dystrophies (OR high myopia 19.5, $P<0.0001$; and OR high hyperopia 10.7, $P=0.033$); rod related dystrophies (OR high myopia 10.1, $P<0.0001$; OR high hyperopia 9.7, $P=0.001$); and RPE related dystrophies (OR low myopia 2.7; $P=0.001$; and OR high hyperopia 5.8; $P=0.025$).

Conclusions

Refractive errors, in particular myopia, are common in inherited RD. Bipolar cells were the common cell type leading to high myopia; *RPGR* and *CACNA1F* were the most common genes associated with high myopia. We determined two critical sites for refractive error development: regulation of glutamate and calcium in the bipolar synapse, bipolar cell neurotransmission, the bipolar routing itself and transport between IS/OS in the photoreceptor. Our findings provide more insight in the various steps in the signaling cascade causing myopia.

INTRODUCTION

Refractive errors (myopia and hyperopia) are the most common ocular disorders worldwide and are a prominent cause of blindness.[1] This highly heritable trait has been subject to many studies, and the search for genes - in particular for myopia - has been ongoing for several decades.[2,3] Myopia is thought to be caused by a visually evoked retina-to-sclera-signaling cascade. Various genes representing different pathways in myopia development have been discovered.[4,5] These pathways include neurotransmission (in photoreceptors, RPE, and choroid), retinoic acid metabolism, extracellular matrix remodeling, and eye development. Still, only ~4% of the heritability of refractive error is uncovered.[5] It is known that refractive errors are common in patients with retinitis pigmentosa (RP, in particular in X-linked forms), congenital stationary night blindness, Stargardt disease and Best macular dystrophy.[6-9] In our outpatient clinic we also became aware of a high presence of refractive errors in patients with other retinal dystrophies (personal communication). Inherited retinal dystrophies are clinically and genetically heterogeneous, causing degeneration of the retinal pigment epithelium (RPE), photoreceptors (cones and rods) or the inner retina.[10] According to the primary location of retinal cell dysfunction, they can be classified into RPE related macular dystrophies, primary photoreceptor dystrophies (cone and rod related dystrophies) and inner retina dystrophies (bipolar cell dysfunctions). Stargardt disease (STGD; 1:10,000) is the most common form of recessively inherited macular dystrophy, caused by mutations in the *ABCA4* gene.[11, 12] Other more rare inherited RPE related macular dystrophies are Best macular dystrophy (BEST) and pattern dystrophy (PD). The subgroup cone related dystrophies is formed by achromatopsia (ACH), a stationary cone disorder, and cone-rod dystrophies (CRD), a more progressive disorder.[13] Rod-cone dystrophies (RCD) also known as retinitis pigmentosa form the largest subtype, accounting for ~ 50% of all inherited retinal dystrophies.[14] Lastly, congenital stationary night blindness (CSNB) is caused by defective retinal signaling from the photoreceptors to the adjacent bipolar cells, and is known to coincide with myopia (OMIM #310500).[15]

Myopia and inherited retinal dystrophies are caused by similar pathways, such as photoreceptor dysfunction and retinoic acid metabolism. Therefore, genes involved in inherited retinal dystrophies are excellent candidate genes for myopia, but their precise roles in the development of refractive errors has never been studied before. Although there are former reports that refer to an increased incidence of myopia in patients with some inherited retinal dystrophies, not all include specific quantifiable data, and they have not included a large spectrum of retinal dystrophies.[6,8,16]

For more understanding in the mechanisms involved in the pathogenesis of refractive error and myopia, we studied a large group of patients with a broad spectrum of inherited retinal dystrophies, classified into RPE related macular dystrophies (RPE), cone related dystrophies (CD), rod related dystrophies (RD), and bipolar cell dysfunction (BPD). We investigated frequency and degree of refractive errors stratified by these four groups of diagnoses and by the specific genes involved in retinal dystrophy.

MATERIAL AND METHODS

Subjects

Our study population consisted of 302 patients with inherited retinal diseases, divided into four groups; RPE related macular dystrophies (RPE, n=77), cone related dystrophies (CD, n=76), rod related dystrophies (RD, n=104) and bipolar cell dysfunctions (BPD, n=45). Patients were ascertained at Erasmus Medical Center, the Rotterdam Eye Hospital, and Bartiméus in The Netherlands, ophthalmogenetic centers which belong to the National RD5000 Consortium. From 2002, patients with ophthalmogenetic diseases who visited the clinic were collected and included in a database. Patients with the clinical diagnosis RPE related macular dystrophy (STGD, BEST and PD), cone related dystrophies (ACH, CRD), rod related dystrophies (RP, RCD) and bipolar cell dysfunctions (CSNB) with available data on refractive error were included. Patients without refractive error data or refractive error data only measured after cataract extraction were excluded. As a reference group, we used 2,940 participants (age 45+ years) from the population-based Rotterdam Study III (RS-III),[17] and 2,610 participants (age 18+ years) from the Erasmus Rucphen Family study (ERF).[18,19]

Clinical assessment

We retrieved all clinical data from medical charts. These data included Snellen visual acuity, objective refraction based on autorefraction or subjective refractive error measured by an optometrist, color vision (Hardy-Rand-Rittler color vision test or Ishihara), visual field tested by Goldmann perimetry and electrical responses of cones, rods and bipolar cells with ERG. For RS-III and ERF (reference group) a similar protocol was used for phenotyping; all subjects underwent an ophthalmologic examination which included non-dilated automated measurement of refractive error (Topcon RM-A2000 autorefractor), best-corrected visual acuity and objective refraction, fundus photography and visual field perimetry (Humprey Visual Field Analyzer, Zeiss, Oberkochen, Germany).

Molecular genetic analysis

In the outpatient clinic, patients with inherited retinal dystrophies were offered DNA analysis for diagnostic testing. DNA was isolated from peripheral blood lymphocytes using standard procedures. *ABCA4* gene mutations were analyzed in CD and RD patients with microarray screening (performed by Asper Biotech, Tartu, Estonia) to assess a safely use of vitamin A therapy. In genetically unsolved retinal dystrophy cases genotyping of the most common mutations in *PROML1, CERKL, CNGA1, CNGB1, MERTK, PDE6A, PDE6B, PNR, RDH12, RGR, RLBP1, SAG, TULP1, CRB1, RPE65, USH2A, USH3A, LRAT* was performed (autosomal recessive RP chip, performed by Asper Biotech, Tartu, Estonia). In patients with RP and deafness suspected for Usher syndrome an additional chip was tested for the most common mutations in *CDH23, MYO7A, PCDH15, Harmonin, SANS, USH2A, VLGR1, USH3a* (USH chip, performed by Asper Biotech, Tartu, Estonia). X-linked CRD or X-linked RCD probands were screened for mutations in the *RPGR* gene. Patients clinically diagnosed with CSNB were screened for mutations in the *CACNAF1* or *NYX* gene.

Statistical analysis

SE was calculated according to the standard formula (SE = sphere + ½ cylinder), and the mean of two eyes was used for analysis. When data from only one eye was available, the SE of this eye was used. In case of multiple SE measurements we used the SE measured during the first visit. We categorized SE into low (SE from -1.5 to -3D), moderate (SE from -3 to -6D) and high (SE of -6 or lower) myopia; emmetropia (SE from -1.5 to +1.5D); and low (SE from +1.5 to +3D), moderate (SE from +3 to +6D) and high (SE of +6 or higher) hyperopia, using previously defined criteria.[20] Analyses were stratified by diagnosis (RPE, CD, RD or BPD) and by causal gene. Causal genes that were found in less than 3 patients were pooled into one group ('other genes'). Logistic regression analyses were used to assess the risk of low, moderate and high myopia and hyperopia versus emmetropia per disease group, using the RS-III and ERF study as the reference group, and adjusting for age and sex. Subgroup analysis (ANOVA tests) were performed to compare the mean SE between different causal genes and to compare differences within *ABCA4* related dystrophies (STGD, CRD and RCD) and within RPE-related dystrophies (STGD, BEST, PD)**.** Since age has an influence on SE (the younger the more hyperopic) we stratified analysis by age (< 25 years; ≥ 25 years) and used the Student *t* test to compare the mean SE between these age groups. All analyses were performed using SPSS version 20 (SPSS Inc.).

RESULTS

Clinical characteristics of the study population (n=302) divided by diagnosis are summarized in Table 1. Of all patients with RPE related macular dystrophies (n=77), most were diagnosed with STGD (n=54), 18 with BEST and only a small group with PD (n=5). In the group with CD (n=76), 15 patients had ACH and 61 patients CRD. The largest group was formed by patients with RD, including 104 RCD patients. With 45 CSNB patients, BPD formed the smallest group.

A DNA sample was available in 83% of all included patients. In 57% of our study population at least one mutation was found. In total, 37% of the cases was genetically solved (autosomal recessive, autosomal dominant or X-linked inherited) (Table 1). Figure 1 shows the frequency of mutations found in various genes within the subtypes of inherited retinal diseases. Most frequently mutations were found in the *ABCA4* gene (36%) (in 33 STGD patients, 19 CRD patients, 10 RCD patients), and in the *USH2A* gene (15%), mainly in RCD patients. Genes that were causing the disease in less than 3 patients were pooled, gene-specific data can be found in Table 2.

The distribution of SE in our patient group and reference group is shown in Figure 2. In CD (mean SE -3.10 D [SD 4.49]), RD (mean SE -2.27 D [SD 4.65]) and BPD (mean SE -6.86 D [SD 6.38]) the distribution was skewed towards the left (more myopic) compared to RPE related macular dystrophies (mean SE -0.10 D[SD 3.09]) and the reference group (mean SE 0.04 D [SD 2.32]). The risks of several degrees of myopia and hyperopia per disease group, compared to the reference group are shown in Figure 3. Persons with bipolar cell dysfunctions had the highest risk of refractive error (OR high myopia 239.7 and OR mild hyperopia 263.2, both $P<0.0001$); followed by cone related dystrophies (OR high myopia 19.5, $P<0.0001$; and OR high hyperopia 10.7, $P=0.033$); rod related dystrophies (OR high myopia 10.1, $P<0.0001$; OR high hyperopia 9.7, $P=0.001$);

Table 1. Clinical characteristics of our study population separated by clinical diagnoses in subtypes

	RPE (n=77)			CD (n=76)		RD (n=104)	BPD (n=45)	RS + ERF (n=5,550)
	STGD	BEST	PD	ACH	CRD	RCD	*CSNB*	Reference
n total	54	18	5	14	62	104	45	5,550
Gender								
Male, n (%)	22 (40.7)	9 (50.0)	1 (20.0)	10 (71.4)	40 (64.5)	52 (50)	42 (93.3)	42 (44.0)
Age								
Mean age of onset, yr (SD)	29.1 (16.9)	26.9 (22.2)	54.2 (10.1)	6.8 (5.75)	18.3 (14.6)	20.3 (17.1)	7.7 (12.7)	NA
Mean age at SE measurement, n (SD)	37.6 (16.2)	37.8 (23.8)	57.8 (10.6)	30.3 (17.6)	33.9 (17.3)	37.6 (16.7)	22.5 (15.8)	53.1 (11.6)
Mean SE (SD)	-1.1 (2.68)	2.3 (2.9)	1.8 (2.5)	-2.9 (5.0)	-3.15 (4.1)	-2.3 (4.7)	-6.9 (6.4)	0.0 (2.3)
DNA sample available, n (%)	51 (94.4)	14 (77.8)	5 (100.0)	13 (92.9)	53 (84.1)	98 (94.2)	18 (40.9)	NA
At least one mutation, n (%)	35 (64.8)	11 (61.1)	3 (60.0)	11 (78.6)	31 (50.8)	68 (65.4)	13 (29.5)	NA
Genetically solved, n (%)	16 (29.6)	11 (61.1)	3 (60.0)	9 (64.3)	20 (33.3)	41 (39.4)	11 (25.0)	NA

RPE =RPE related macular dystrophies; CD = cone related dystrophies; RD = rod related dystrophies; BPD = bipolar cell dysfunctions; STGD=Stargardt disease; BEST=Best macular dystrophies; PD=pattern dystrophy; ACH=achromatopsia; CRD=cone-rod dystrophies; RCD=rod-cone dystrophies; CSNB=congenital stationary nightblindness; SD=standard deviation; SE=spherical equivalent; NA, not applicable.

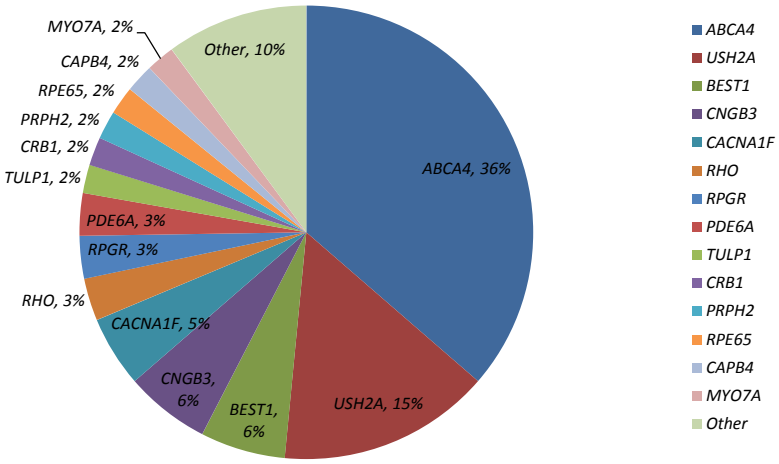

Figure 1. Frequency of causal genes in our study population (n=172).
The group "other" consists of various genes found in less than 3 subjects

Table 2. Distribution of spherical equivalent in 'other genes'
"Other genes", genes with causal mutations found in less than 3 subjects, were pooled.

Other genes	n = 17	SE
ALMS1	1	6.81
BBS5	1	-0.06
CERKL	1	-0.13
CNGA1	1	-2.50
CNGA3	1	-8.50
EYS	2	-4.75 ; -0.88
GUCY2D	2	-23 ; -3.50
IMPG2	1	-6.38
KCNV2	1	-3.25
NYX	1	-11.00
PDE6B	1	0.25
PDE6C	1	12.00
PRPF8	1	-0.38
TRPM1	1	-8.31

n=number; SE= spherical equivalent

and RPE related dystrophies (OR low myopia 2.7; *P*=0.001; and OR high hyperopia 5.8; *P*=0.025). A subgroup analysis within the RPE related macular dystrophies showed a significant (*P*< 0.0001) difference in mean SE between STGD patients (n=54; mean SE -1.10 D [SD 2.68]) and BEST patients (n=18; mean SE 2.34 D [SD 2.91]).

Table 3 shows the mean SE stratified by age at first visit. Mean age at first visit in our study population (n=302) was 35 years [SD 18], ranging from 0 to 80 years and differing between subtypes. Only patients with rod related dystrophies had a significantly (*P*=0.027) lower mean SE (-2.83 D [SD 4.65]) in patients >25 years than patients ≤ 25 years of age (mean SE -0.49 D [SD 4.22]).

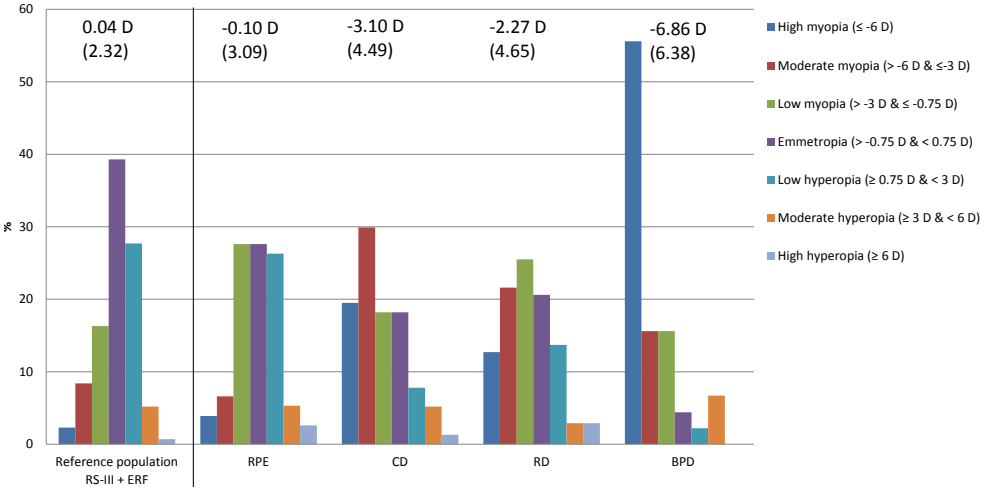

Figure 2. Refractive error distribution in retinal dystrophies
Percentages of subcategories of spherical equivalent are shown for patients with RPE-related dystrophies (RPE), cone related dystrophies (CD), rod related dystrophies (RD) and bipolar cell dysfunctions (BPD), compared to our reference group (RS-III and ERF). Mean SE in diopters (D) ± standard deviation are shown above each subgroup.
RPE=RPE-related dystrophies; CD=cone related dystrophies; RD=rod related dystrophies; BPD=bipolar cell dysfunctions; RS-III= Rotterdam Study III; ERF = Erasmus Rucphen Family study

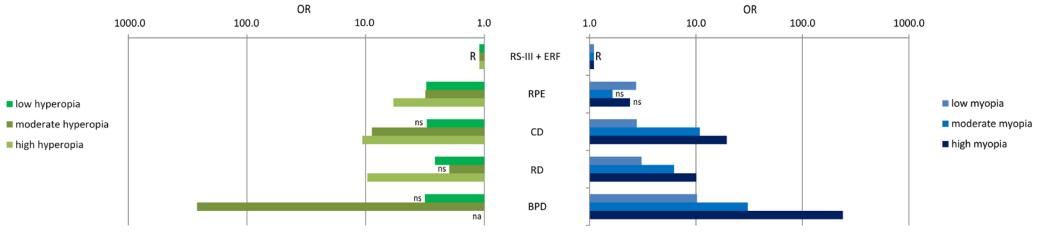

Figure 3. Risk of myopia and hyperopia per primary affected cell type
This plot shows odds ratios of several degrees of myopia (right) and hyperopia (left) versus emmetropia per disease group. The RS-III and ERF study were used as the reference group (R), and analyses were adjusted for age and sex. The BPD group was too small to calculate an odds ratio for high hyperopia (na). OR, odds ratio; RS-III, Rotterdam Study III; ERF, Erasmus Rucphen Family Study; RPE, retinal pigment epithelium related macular dystrophies; CD, cone related dystrophies; RD, rod related dystrophies; BPD, bipolar cell dysfunction; na, not applicable; ns, non significant.

Table 3. Spherical equivalent stratified by age

	<10 yrs		10-25 yrs		>25 yrs		P-trend
	n	mean SE (±SD)	n	mean SE (±SD)	n	mean SE (±SD)	
RPE (n=77)	5	3.9 (2.9)	15	-0.86 (1.78)	57	-0.25 (3.15)	0.292
CD (n=76)	8	-0.23 (7.92)	20	-2.86 (3.16)	48	-3.68 (4.13)	0.023
RD (n=104)	4	1.94 (2.23)	21	-0.95 (4.39)	79	-2.83 (4.66)	0.076
BPD (n=45)	11	-3.84 (4.17)	18	-6.52 (6.28)	16	-9.34 (7.05)	0.959

RPE = RPE related macular dystrophies; C(R)D = cone related dystrophies; R(C)D = rod related dystrophies; BPD = bipolar cell dysfunctions; SE = spherical equivalent; SD = standard deviation. P-value calculated with students T-test, P < 0.05 was considered statistically significant

In Figure 4A, the spherical equivalent distribution is shown for various genes causing inherited retinal dystrophies. Most genes (9 out of 14) coincided with myopia. With a mean SE of -2.02 D [SD 3.71] the *ABCA4* gene formed the most common disease causing gene (n=62). The *ABCA4* gene, causing the three different diagnoses Stargardt disease (SE -1.50 [SD 2.85]), cone-rod dystrophy (SE -2.72 [SD 4.11]) and rod-cone dystrophy (SE -2.41 [SD 5.31]), showed no significant differences ($P = 0.493$) in SE. Patients with mutations in the *BEST1* gene (n=11) had a mean SE of 1.84 D [SD 2.95], which is significantly higher than the mean SE of the *ABCA4* (n=62; $P = 0.051$) or the *RPGR* gene (n=6*;* $P = 0.001$). Patients with mutations in the *RPGR* gene (n=6) were the most myopic (mean SE -7.63 D [SD 3.31) of all.

DISCUSSION

We studied refractive error in 302 patients with inherited RD, and found that genes involved in these diseases often coincide with refractive error. Our results show that in particular BPD is associated with both high myopia and hyperopia, and CD and RP on the whole lead to mild myopia. This suggests that in particular bipolar cells are crucial for the emmetropization process.

Although systematic reports are lacking, occasional case series reported refractive errors in patients with RD. Mild refractive errors have been described for Stargardt and Best disease[6,9]; more severe refractive errors for CSNB and for RP caused by *RPGR*.[8, 16, 21-23] Our comprehensive analysis shows that RPE related dystrophies such as Stargardt, Best and pattern dystrophy indeed mostly have mild refractive errors. Photoreceptor disorders have more variability in refractive errors, but are also mildly myopic on average; CD have a somewhat higher frequency of moderate myopia (SE from -3 to -6D) than RP. Our study confirms that BPD predominantly leads to severe refractive errors, and remarkably, the direction of the SE is dependent on the causal gene.

Our study has a unique design in myopia research. We selected patients with a known primary defect in the retina, and studied the effect on refractive error. We focused on affected cell types as well as on causal genes within each cell type. Since myopia develops at childhood and early youth, we stratified for age and found differential effects even in those with a very early onset. Our study is the only systematic investigation on refractive error and RD to date and includes a broad spectrum of diagnoses. In addition, we had access to large population-based studies, which we could use as a reference population. Our study has some drawbacks as well. Although our total sample size was substantial, subgroup analyses were limited by small numbers. The reference population was somewhat older that he RD cases, however, comparison to the younger ERF population did not significantly alter the risk estimates (data not shown). Lastly, our RD patients lacked data on established risk factors for myopia such as familiar occurrence of myopia and educational level.

Given our findings, what can we learn from retinal dystrophies about the development of myopia? Figure 4B shows the localization of the gene products in the retina. Bipolar cells appear to have a crucial role in emmetropization. Bipolar cells play an essential role in the retinal microcircuitry and transmit signals from photoreceptors to ganglion cells.[24] They can be divided by ON and OFF subtypes, which determine excitatory and inhibitory responses in reaction to light exposure. Rod signal transmission primarily occurs through ON bipolar cells, whereas cones connect with both

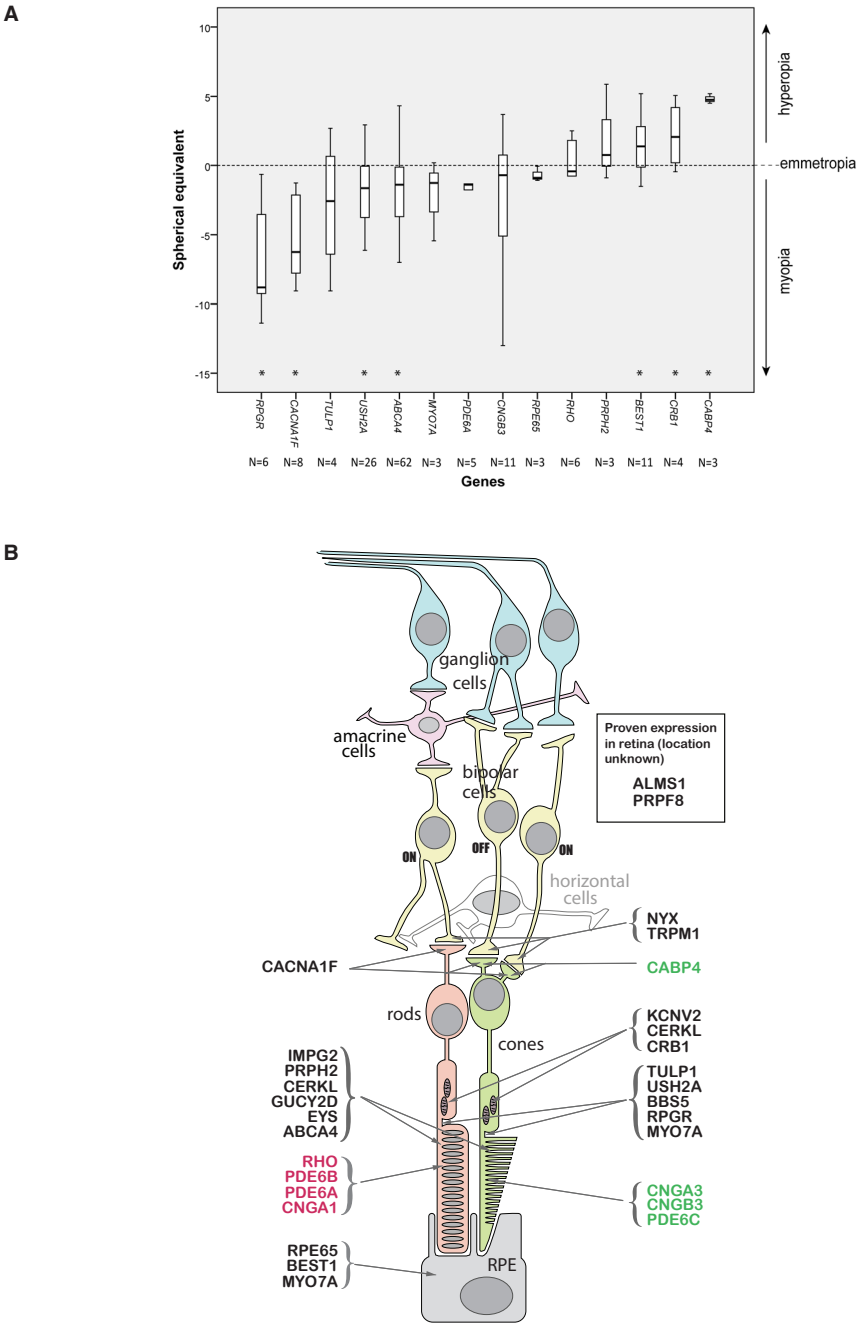

Figure 4. Distribution of refractive errors for gene-specific inherited retinal dystrophies
A. Boxplots of the distribution of spherical equivalent (in diopters) on Y-axis per specific causal gene on X-axis. Causal genes that were found in less than 3 patients were not included in this figure, but their SE measurements can be found in Table 2.
B. Localization of retinal dystrophy gene products in the retina. In green: cone specific genes. In red: rod specific genes.

ON and OFF bipolar cells. If we take a closer look at genes causing BPD, our study shows that mutations in *CACNA1F*, *NYX* and *TRPM1* lead to high myopia, whereas mutations in *CAPB4* lead to high hyperopia. These genes all function at the photoreceptor-bipolar synapse, and determine the glutamate and calcium dependent neurotransmission of the bipolar cell.[25,26] *TRPM1* and *NYX* are both localized at the postsynaptic terminal of the ON bipolar cells, and are involved in an intracellular signaling cascade.[26] *CACNA1F* is a presynaptic calcium channel; *CAPB4* is also expressed presynaptic, but binds calcium in the synaptic space. *CACNA1F* is present in both photoreceptor types, while *CAPB4* is only present in cones.[26-28] Mutations in all genes cause an electronegative waveform response, but only mutations in *CABP4* lead to photophobia rather than nyctalopia, and is considered a cone synaptic disorder.[29] These considerations suggest that regulation of glutamate and calcium in the bipolar synapse, bipolar cell neurotransmission, and the bipolar routing itself may all be mechanisms in refractive error development.

Several photoreceptor genes show a relation with myopia, while others have virtually no effect or show a relation with hyperopia. Mutations in *RPGR,* which is located in the connecting cilium and is involved in micro-tubular transport, cause high myopia, whereas mutations in *USH2A*, which is located at the apical side of the inner segment and is responsible for the maintenance of the photoreceptor, cause mild myopia. The development of myopia seems to be dependent of the location of the gene product in the photoreceptors; some structures seem to be more vulnerable for development of refractive error. A recent study on RP mice with *PDE6B* mutations suggests that changes in refractive error could be due to alterations in dopamine metabolism.[30]

Previously, numerous myopia susceptibility loci were identified for refractive error in population-based studies.[4,31] Some of the novel associations were in or near genes involved in the visual cycle and retinoic acid metabolism, i.e. common variants in *RDH5* (encoding retinol dehydrogenase 5) and *RGR* (encoding the retinal G protein coupled receptor).[4,31] Mutations in *RDH5* cause a rare form of congenital stationary night blindness and progressive cone dystrophy[32,33], and mutations in *RGR* are involved in retinitis pigmentosa.[34,35] Another myopia susceptibility gene that was identified was *CACNA1D* (encoding a voltage-sensitive calcium channel regulator).[31] This gene shows great similarity with the *CACNA1F*-gene. Both genes encode a protein of the alpha subunit in the L-type calcium channel, localized in the presynaptic ribbon terminals of photoreceptors. Lastly, one of the newly identified genes, *GRIA4* (encoding glutamate receptor, ionotropic, AMPA 4) encodes a glutamate-gated ion channel that mediates fast synaptic excitatory neurotransmission, is present in the retina and is crucial for emmetropization.[36-39] We hypothesize that mutations in these RD genes can cause retinal dystrophies, whereas other - less damaging – more common genetic variants within these genes can cause myopia only.

In conclusion, we showed that most genes involved in retinal dystrophies coincide with myopia, especially those causing BPD, CD and RP. We determined two critical sites for refractive error development: regulation of glutamate and calcium in the bipolar synapse, bipolar cell neurotransmission, and the bipolar routing itself and transport between IS/OS in the photoreceptor

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6.1

General Discussion

GENERAL DISCCUSION

The aim of this thesis was to expand our knowledge regarding the pathogenesis and clinical impact of myopia and refractive error. Our studies provide important data with respect to the prevalence, impact, and genetic and environmental risk factors associated with myopia. This general discussion will highlight our most important findings and place them in the context of disease etiology and disease risk. We will also discuss the next logical steps towards further understanding the pathogenesis of myopia.

MAIN FINDINGS AND CLINICAL RELEVANCE

Burden of disease

In **Chapter 2**, we examined the burden due to myopia and refractive error. We studied (1) the prevalence of refractive error in Europe (Chapter 2.1); (2) the relationship between refractive error and visual impairment (Chapter 2.2); and (3) the relationship between axial length and visual impairment and (Chapter 2.3).

Chapter 2.1 discusses the prevalence of myopia in Europe. Although the prevalence of myopia and refractive error in developed countries has been studied extensively[1,2], estimates of refractive error in Europe were relatively outdated and were based only on a single cohort[3]. Therefore, we measured the prevalence of refractive error in nearly 62,000 participants pooled from population-based studies in the European Eye Epidemiology (E^3) consortium (Chapter 2.1). We found that refractive error affects more than half of all adults in Europe. The most common refractive error is myopia, with prevalence rates of 30% for myopia and 3% for high myopia. The highest prevalence was found among young adults; nearly 50% of this population is myopic, confirming that the prevalence of myopia is increasing in younger generations[4-7].

In Chapter 2.2, we studied the causes of blindness and low vision in relation to refractive error. It is generally accepted that high myopia often leads to vision-threatening complications[8-10]. However, up-to-date prevalence rates regarding myopic macular degeneration, glaucoma, and retinal detachment, as well as the precise risk of visual impairment among persons with high myopia, were not available. We therefore examined the frequency and causes of blindness and impaired vision in the population-based Rotterdam Study; these data were stratified for various refractive error categories. We found that one-third of all individuals with high myopia develop severe bilateral visual impairment, and this impairment is caused primarily by myopic macular degeneration. The risk of impairment increases with each incremental increase in refractive error: thus, compared with emmetropic individuals high myopes with a spherical equivalent (SE) of -6 D to -10 D have a 3.4-fold increased risk of visual impairment; high myopes with an SE of -10 D or worse are have a 22-fold increased risk .

In Chapter 2.3, we further explored the risk of visual impairment in relation to axial length (AL) in a combined dataset from the population-based Rotterdam Study, the family-based ERF study and the case-control study MYST. The risk of visual impairment in high myopes was highly correlated with AL. Strikingly, the lifetime risk of visual impairment in eyes with an AL>30 mm was >90%. Eyes

with AL 26-28 mm gradually developed VA from 60-70 years, whereas eyes with >28 mm began to develop visual impairment as early as 45 years of age. These alarming results are consistent with previous studies that found a higher prevalence of pathological signs such as axial length and/or refractive error[10-13]. Moreover, our data presented in Chapter 2.2 and 2.3 provide valuable additional information, as we examined large cohorts, thus enabling us to perform a robust calculation of the risk of visual impairment in a great range of refractive error and axial length categories.

Overall, our data regarding the burden associated with myopia clearly illustrate how myopia is a growing public health concern, affecting Western countries as well as Asian populations. Globally, an estimated 2.5 billion people will be myopic in the next decade, and the estimated annual costs of lost productivity due to visual impairment from refractive error is $268 billion[14]. Moreover, in Singapore alone, the annual costs associated with treating myopia-related complications has been estimated to reach $2.5 million[15]. The current paucity of adequate treatment modalities and this dramatic rise in the number of new high myopes—including here in the Netherlands—will place a significant burden on both our public health and our economy[1,14,15]. Our data underscore the need for a proper treatment, as reducing progressive eye growth to achieve a lower final axial length would significantly decrease the patient's risk of developing visual impairment later in life. Indeed, each incremental decrease in final AL will improve the patient's long-term prognosis in terms of preserving visual acuity.

Genetics of refractive error and myopia endophenotypes

Research in recent decades has shown that heritable factors play a key role in ocular refraction[16-21]. However, it was not until the introduction of high-throughput genome-wide genotyping that dissection of the disease genes became possible. Genome-wide association studies (GWASs) and other genomics-based technologies have accelerated the discovery of genes and genomic regions that contribute to complex genetic disorders.

The first genomic hits for common refractive errors were discovered by our group (Chapter 3.1) in collaboration with the Twins UK Study[22]. Two loci were identified on chromosome 15, and the closest genes in these regions are *GJD2* and *RASGRF1.* Identifying the functions of these genes led to new hypotheses regarding myopia development, as both genes play a role in retinal neurotransmission. The *GJD2* gene encodes a protein that forms gap junctions between neurons in the retina, enabling the intercellular exchange of small molecules and ions[23-26]. The *RASGRF1* gene encodes a nuclear exchange factor that is involved in the transmission of photoreceptor responses[27,28]. Identifying these loci provided the first clear evidence that neurotransmission in the retina plays a role in myopia development.

In 2011, we initiated a large consortium called CREAM (the Consortium for Refractive Error And Myopia), which combined data regarding refractive error and genetic markers from 56 international studies. Using this large dataset, we confirmed our hit on chromosome 15q14 (Chapter 3.2). In 2013, we performed a large-scale GWAS meta-analysis of spherical equivalent within the CREAM consortium. In addition to replicating both hits on chromosome 15, we found genome-wide significance for an additional 24 loci in 45,758 individuals (Chapter 3.3). The risk of

myopia among individuals carrying the highest number of risk alleles is ten-fold higher than the risk among individuals with an average number of risk alleles. We also found that some of the loci identified from CREAM (Chapter 3.3) and 23andMe[29] studies are associated with refractive error as a dichotomous trait (Chapter 3.4).

At nearly the same time, the commercial direct-to-consumer genetic testing company 23andMe identified 22 genomic loci in 45,771 individuals using data obtained from questionnaires regarding the diagnosis of myopia and the age of first glasses as outcome variables[29]. Despite using a different phenotyping method, the results obtained from the 23andMe study were strikingly similar to the results obtained by the CREAM consortium; specifically, 12 genome-wide significant hits overlapped (Figure 1). In addition, the effect sizes of most of the associations were linearly correlated[30], indicating that these genetic associations were robust and can likely be generalized to other populations. The striking similarity between Caucasians and Asians in terms of genetic associations strengthened this notion. Other GWASs of high-myopia case-control cohorts of Han Chinese ethnicity found evidence of an association with several candidate genes[31-34]. Among these associations, only the association with the *CTNND2* gene at 5p15 (identified by Li et al.[34]) was confirmed by other Asian studies[35-37]. None of these loci could be confirmed in the population-based GWASs from either CREAM (Chapter 3.3) or 23andMe[29], suggesting that these loci likely represent Han Chinese and/or family-specific genetic factors.

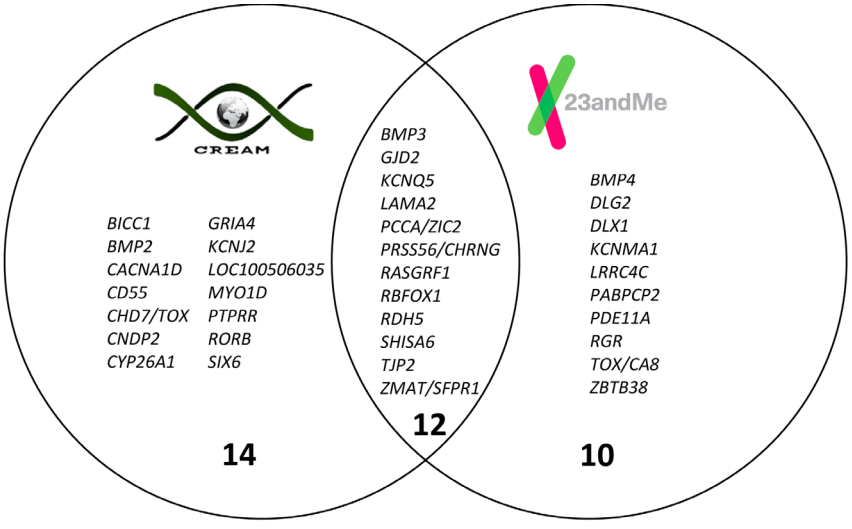

Figure 1. Venn diagram of genes associated with spherical equivalent and myopia from the CREAM and 23andMe studies

The CREAM consortium also provided the opportunity to perform GWASs of other biometric phenotypes of the eye. In a subset of the CREAM population, we identified nine loci for axial length (Chapter 3.5), one of which had been identified previously[38]. Interestingly, among these nine loci for axial length, only three—the *GJD2*, *LAMA2*, and *CD55* genes—were also associated with spherical equivalent. On the other hand, the locus with the highest association with axial

length—the *RSPO1* gene—was not associated with refractive error in this study. This discrepancy in associated genes could be due to false positive findings for either spherical equivalent or axial length. Another possible explanation may be the smaller sample size in our GWAS meta-analysis of axial length, in which we may have lacked sufficient power to detect the overlapping associations with spherical equivalent. Moreover, because the primary determinants of refractive error are axial length and corneal curvature, each of which has its own genetic distribution[18,39,40], the missing genetic overlap between refractive error and axial length could be explained—at least in part—by overlapping genetic factors for corneal curvature. A GWAS meta-analysis of corneal curvature in CREAM is ongoing, following previous studies of this phenotype[41,42], and this analysis will hopefully shed new light on this issue. In addition, multiple-trait analyses may provide insight into the pleiotropic effects of single-nucleotide polymorphisms (SNPs) associated with spherical equivalent, axial length, and corneal curvature[43-45].

We also studied the genetic susceptibility of a third refractive phenotype, astigmatism, which is currently poorly understood. Previously published GWAS meta-analyses of large numbers of individuals derived primarily from population-based cohorts identified an association with a single locus[46,47]. Even in our CREAM GWAS meta-analysis of refractive astigmatism, which included the largest study population for this phenotype examined to date, we only identified putative candidate genes for refractive astigmatism (Chapter 3.6). These results could suggest that most of the additive genetic risk for astigmatism arises from the combined effect of many individual risk variants, each of which has a small effect. Alternatively, it should be noted that the axis was not taken into account in our analyses. Among younger individuals, astigmatism generally goes "with the rule", whereas astigmatism generally while in later life it usually switches to "against the rule" in older individuals[48,49]. A loosening of eyelid tension is the most widely supported theory explaining this change[50]. Thus, if against-the-rule and with-the-rule astigmatism have different etiologies, future GWAS analyses should achieve maximum statistical power by analyzing these two types of astigmatisms separately, and they should stratify their analyses by age. A third possibility for the lack of more genetic hits for astigmatism could be the case-control design of the study; a quantitative endophenotype—rather than a binary trait—can be used in future analyses to increase power.

From the genes identified in **Chapter 3**, we can annotate several pathways associated with refractive error, myopia, and axial length (Table 1; Figure 2). These pathways are consistent with the current hypothesis regarding myopia pathogenesis: a visually evoked signaling cascade (neurotransmission, signaling, retinoic acid genes) originates in the retina (neuronal development, ganglion cell genes), traverses the retinal pigment epithelium and choroid (signaling, intracellular movement genes), and terminates in the sclera, where active remodeling of the extracellular matrix (extracellular matrix genes, retinoic acid, apoptosis genes) causes an elongation of the eye (Figure 3). These neuronal development–related genes may exert their effects at multiple sites in this signaling cascade.

Table 1. Genes identified for spherical equivalent and axial length annotated to genetic pathways
Genes identified by studies incorporated in this thesis are shown in **bold.** Genes with a * are associated with both spherical equivalent and axial length.

	Genes	Pathway / Function
Spherical equivalent, myopia, and hyperopia	***GJD2*, RASGRF1***	Neurotransmission
	KCNQ5, CACNA1D, KCNJ2, *KCNMA1*	Ion channels
	RBFOX1, CHRNG, PDE10A, *GPR25, PDE11A*	Signal transduction
	PDE11A, PTPRR	MAPK signaling
	BICC1*, SFRP1, TCF7L2*	Wnt signaling
	CNDP2, *MIPEP, NPLOC4, PZP, B4GALNT2*	Protein processing
	RDH5, CYP26A1, MAP2K, *RGR*	Retinoic acid metabolism
	LAMA2*, BMP2, BMP3, *BMP4, ADAMTSL1, UND*	Extracellular matrix remodeling
	GRIA4, ARID, GABRR1	GABA / glutamate signaling
	TJP2, *LRFN5*	Cell adhesion
	SIX6, PRSS56, CHD7, CTNND2, RORB, *DLX1, ZNF64*	Eye development
	ZIC2	Ganglion cell growth
	CD55*, *C1QTNF9B*	Complement cascade
	ZMAT4	DNA binding
	AREG	Cell growth
	MYO1D	Intracellular movements
	LRRRC4, DLG2	Neuronal development
	BLID	Apoptosis
	SHISA6, TOX, LOC100506035, SLC14A2, LINC00340, FAM150B, *BI480957, CA8, EHBP1L1, PABPCP2, QKI, SETMAR, SH3GL2, TMEM98, ZBTB38*	Unknown
Axial length	***GJD2****	Neurotransmission
	LAMA2*	Extracellular matrix remodeling
	CD55*	Complement cascade
	RSPO1, ZNRF3	Wnt signaling
	ZC3H11B	RNA binding and processing
	C3orf26, ALPPL2, TIMELESS	Unknown

Environmental factors and gene-environment interactions

In **Chapter 4,** we discuss environmental factors and gene-environment interactions with respect to the pathogenesis of myopia. Many studies found that lifestyle factors play a key role in the onset and progression of myopia[51,52]. Indeed, compelling evidence suggests that the rising prevalence of myopia can be attributed largely to environmental factors that are associated with increased education and urbanization[53]. Importantly, education has a particularly high association with myopia; specifically, individuals with university or high vocational education have a 5-8-fold higher risk of having myopia than individuals who completed only primary school[53,54]. In Chapter 4.1, we investigated the rising prevalence of myopia throughout Europe, and we asked whether rising education levels might explain this trend. We found that higher education is an additive factor—rather than an explanatory factor—in this cohort effect. Similar effects have been observed with respect to urban areas versus rural areas, with urban regions having a significantly higher prevalence of myopia[55].

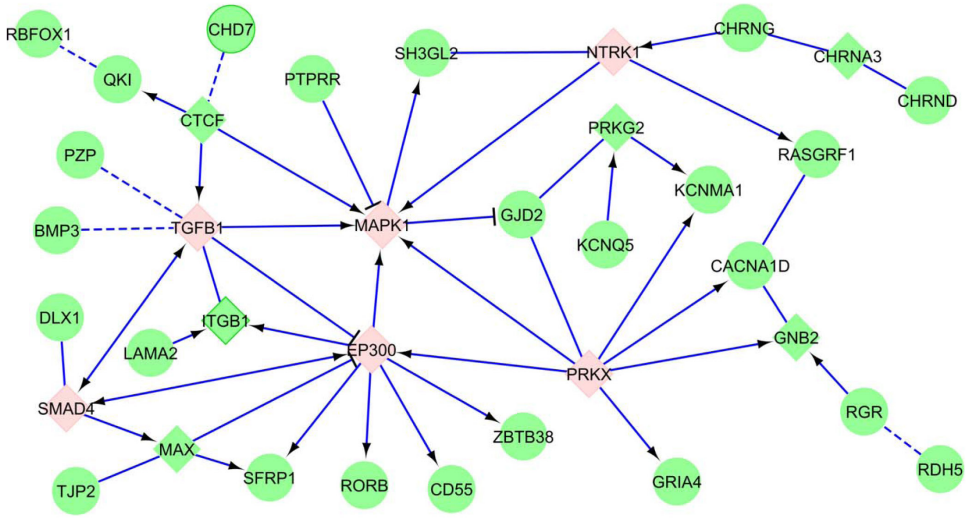

Figure 2. Pathway analysis for myopia genes
Adapted from Hysi et al.[113] Network connections of genes associated with refractive error in CREAM (Chapter 3.3) and 23andMe [29]. The genes directly identified in these GWAS analyses are shown in round nodes, and the linker elements are shown in diamond-shaped nodes. Key MAPK, TGF-beta/SMAD pathway elements are shown in pink. Solid blue lines depict known protein–protein interactions; dashed blue lines depict co-regulation relationships. The network was constructed using the Reactome database[114,115].

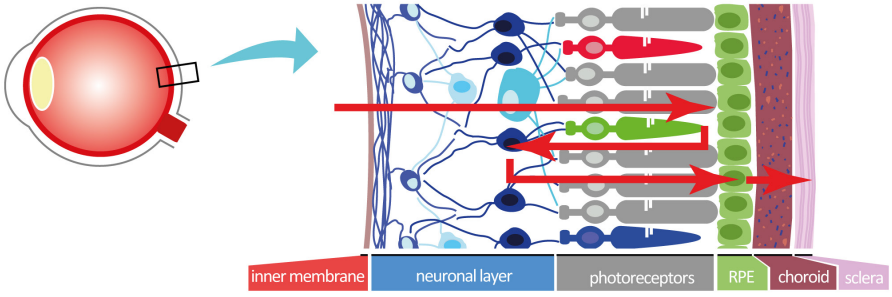

Figure 3. Schematic model of the myopia signaling cascade

The association between these environmental factors and myopia has been investigated, and two key observations have emerged: (1) myopic children generally spend less time outdoors than non-myopic children, and (2) myopic children perform more near work at an earlier age compared to non-myopic children[56-58]. The putative protective effect of spending more time outdoors is believed to be due to light intensity[59]; indoor illumination is approximately 500 lux, whereas outdoor light levels generally exceed 20,000 lux during the day. Higher light intensity has been associated with higher dopamine release in the retina[60], and animal studies have shown that increased dopamine

levels can slow elongation of the eyeball[61]. Dopamine is released in a light-dependent fashion, but its release also depends on the spatial feature of the image[62]. It is possible that the spatial tuning of the retinal neurons determines the signals for eye growth[61].

The association between near work and myopia is less clear, as the type of near work is a difficult factor to study, and other factors—such as using handheld digital devices and reading—have led to inconsistent and/or poorly reproducible results[63,64]. One current hypothesis is that near work induces myopia due to long periods of defocus in the peripheral retina[65], particularly in eyes that are prolate in shape (Figure 4)[66].

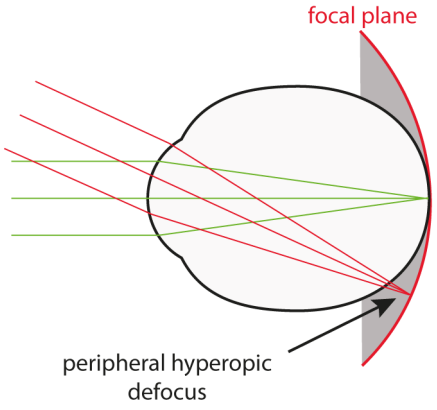

Figure 4. Peripheral hyperopic defocus
Prolate-shaped eyes have a relatively high depth of hyperopic defocus in the periphery. This may trigger elongation of the eye.

Of all the risk factors for myopia identified to date, education is by far the most easily obtained, has robust variables, and is not likely to lead to misclassification. On the other hand, cultural differences in educational systems can make it difficult to compare studies performed in different parts of the world. In addition, many studies used years of study as a measure of education, without taking into account the intensity of the study[53]. Lastly, educational level has generally been considered a surrogate measurement for cumulative near work activity, and one could argue that better results would likely be obtained by measuring near work activity directly.

Having reviewed the advantages and disadvantages of studying education as an environmental factor, given that myopia likely results from interactions between the effects of genes and the environment, a study of combined effects is clearly warranted. We therefore studied the role of education in the development of myopia, and we performed gene-by-environment interaction analyses. We found an interaction between education and genetic risk; specifically, patients with both high genetic load and a university-level education had a much higher risk of myopia than patients with only one of these two factors (Chapter 4.2). This interaction was specific to three genetic loci, *SHISA6-DNAH9*, *GJD2*, and *ZMAT4-SFRP1*[67].

In Chapter 4.3, we used a gene-environment-wide interaction study (GEWIS) to test the joint

contribution of the main effect of the SNP and SNP-by-education effects. Using this approach, we identified ten novel loci associated with refractive error in our CREAM cohort. These findings demonstrate the value of applying the joint methods recently proposed by Manning et al.[68] by incorporating a clearly associated environmental risk factor in order to identify novel genetic risk factors, thereby shedding light on the mechanisms that lead to myopia. The novel genetic loci associated with refractive error could not have been identified using standard GWAS approaches. Our studies gene-by-environment interactions suggest that environmental factors are the requisite trigger for gene expression, thereby causing myopia. This interaction likely accounts for the recent increase in prevalence in association with increased education and urbanization.

Interestingly, our GEWIS approach revealed strong interaction effects only in the Asian cohorts. This could imply that interaction effects in Caucasians may be too small to be detected using these methods, for example due to high variability among educational systems in Western countries. As discussed above, education may not be an accurate surrogate for measuring cumulative near work activity among Caucasian adults. We also propose that the strong interaction effects measured in Asian cohorts may underlie the remarkably higher prevalence of myopia in Asian countries compared to Western countries.

Functional mechanisms of myopia

Studies of refractive error in a variety of animals—including chickens, rats, mice, marmots, guinea pigs, and monkeys—have laid the foundation for our understanding of the effect of visual input on eye growth. Several experimental strategies have been used to induce myopia in animals, including form deprivation (by blurring the eye), visual deprivation (by suturing the eyelid closed)[69-72], and placing a negative (i.e., concave) lens in front of the eye[73-76]. Placing a positive lens in front of the eye counteracts myopia by slowing eye growth[77]. These lens-induced effects appear to be independent of visual transmission to the brain, as they are also observed in animals with a disrupted optic nerve. Recent animal studies demonstrated that the peripheral retina is more responsive to blur than the macula[66,78], and experiments with monkeys and chickens support the notion that peripheral retinal defocus is a stimulus for the onset of myopia[66,79].

One question that arises from these animal studies is the retinal cell type(s) that are responsible for the development of myopia. Many genes that were found to be associated with spherical equivalent in our GWASs played a role in retinal neurotransmission, suggesting that photoreceptor cells may be important in myopia development. To address this question further, we were fortunate to have access to ophthalmogenetic clinics to study a large series of retinal dystrophy patients. These patients mostly have a Mendelian cause to their disease, affecting only one retinal cell type as the primary site. In **Chapter 5**, we studied refractive error in a group of 302 patients with a variety of inherited retinal dystrophies; our results confirmed previous reports that the prevalence of refractive error is significantly higher in these patients[80-87]. Patients with a cone dystrophy or retinitis pigmentosa generally present with mild myopia. However, patients with congenital stationary night blindness (CSNB)—which is caused by defective signaling from photoreceptors to the ON-bipolar cells in the retina—often present with either high myopia or high hyperopia. Bipolar cells play an essential role in the retinal microcircuitry, transmitting signals from photoreceptors

to ganglion cells[88]. Bipolar cells can be divided into the ON subtype and the OFF subtype based on whether the cells have an excitatory or inhibitory response to light exposure. Rod cells signal primarily through ON-bipolar cells, whereas cone cells signal via both ON-bipolar cells and OFF-bipolar cells[89]. This finding sheds new light on myopia pathogenesis and suggests that we should also focus on ON-bipolar cells as a key player in the development of myopia.

METHODOLOGICAL CONSIDERATIONS

Relevant methodological issues have been addressed in the respective discussion sections of each individual chapter. Here, we will highlight some of the general methods and issues that we encountered.

Phenotyping issues

Interpreting the evidence available regarding the prevalence and pathogenesis of myopia and refractive error can have several issues. In this thesis, we mainly used spherical equivalent to analyze refractive error as a quantitative trait, as data regarding the sphere and cylinder are clinically relevant and were readily available for all cohorts. The disadvantage of using either refractive error or spherical equivalent is that they are composite variables determined primarily by axial length and corneal curvature[18,39,40]. Thus, analyzing axial length (AL), corneal curvature (CC), or the AL/CC ratio may yield more objective, precise, and reproducible results compared to analyzing refractive status. However, AL and CC are not commonly measured in clinical practice and were not measured in our study settings. Other factors that were not addressed in this thesis are peripheral refraction and the shape of the eyeball.

With respect to analyzing dichotomous or categorical variables, significant differences exist in the definitions of myopia and high myopia[7,40,53]. Myopia is commonly defined as an SE of –0.5 D or worse, whereas high myopia is defined (rather arbitrarily) using a cutoff that ranges from –5 D to –10 D. Fortunately, throughout this thesis and including the consortium papers arising from the E^3 and CREAM studies (Chapters 2.1, 3.2, and 3.3), we used consistent thresholds for myopia and high myopia (–0.75 D and –6 D, respectively).

Genome-wide association studies

In Chapter 3, we used the GWAS approach to identify genetic associations for several refractive phenotypes. GWAS approaches have successfully identified hundreds of genetic variants associated with complex human diseases and traits, and they yielded valuable insights into their genetic architecture[90]. The main advantage of a GWAS is that it offers a hypothesis-free alternative that is often more appropriate for the genetic dissection of complex traits that are affected by several genetic variants. A large GWAS can have the statistical power needed to yield highly reliable results, and it can provide the firm foundation needed to draw the first lessons from genetic analyses of a complex genetic trait. However, the use of a GWAS can have its limitations as well.

First, if multiple genes are involved in a trait, large sample sizes and replication studies are needed in order to detect an association. This requires the formation of collaborative consortia in order to recruit sufficient numbers of participants for meta-analyses. A

drawback of these consortia is that they consist of many cohorts, thereby increasing heterogeneity among the phenotypes and genotypes being studied. Thus, we were able to identify only highly consistent SNP effects in all cohorts. Due to geographical differences between cohorts, specific variants might exist but may not have been detected.

Second, the alleles identified using a GWAS are usually not the causative alleles but are in linkage disequilibrium with the true causative variants. Identifying the causal variant in an associated locus can be difficult due to this linkage disequilibrium or because most GWAS loci contain multiple genes (or no genes at all). Relatively few of the proposed candidate genes have been experimentally validated. Indeed, the only way to show causality is to determine the biological pathway and the precise role that the gene plays in producing the trait or disease. Because we identified associations with quantitative effects, it might not be necessary to identify the precise causal variant underlying all identified associations. At minimum, we must investigate the functional role of the gene in order to reliably identify the pathway(s) involved. This is particularly true for genetic variants that are located in gene deserts in the vicinity of specific genes. Thus, the results obtained from a GWAS should always be interpreted using functional data.

Lastly, the genetic variants identified by our study explain only a small fraction of the total heritability of these traits. Despite the high number of loci identified to date, only approximately 12% of the phenotypic variance is explained (Verhoeven et al., ARVO 2014, unpublished data). Given that the expected estimate of the total heritability of refractive error is approximately 71%[17], much of the heritability underlying refractive error and myopia is still missing. There are two plausible explanations for this finding: (1) we focused on common variants, and rare variants are still undiscovered[91]; or (2) gene-gene and gene-environment interactions determine some of the variance[92,93]. Below, we discuss strategies for identifying risk variants and for further identifying this missing heritability.

Due to the above-mentioned limitations, GWAS approaches have been met with considerable skepticism with respect to their clinical applicability. Nevertheless, several GWAS findings have been translated successfully to clinical applications, including risk prediction, disease classification, drug development and drug toxicity[94]. Moreover, our studies have revealed putative pathways underlying myopia and refractive error; these pathways would not have been identified using other approaches. These results will serve as the starting points for future research. Translating these findings into direct health benefits will require an interaction between many biomedical disciplines, including genomics, molecular biology, bioinformatics, clinical medicine, and pharmacology[94,95], and will be discussed in the next paragraph.

FUTURE DIRECTIONS

The studies described in this thesis represent an important step towards identifying genetic associations, and they provide important hints for potential pathways. Future research should focus largely on issues regarding methodological considerations, including (1) studying other endophenotypes of myopia and refractive error, including peripheral refraction and eyeball

shape; (2) identifying the missing heritability by identifying rare variants, gene-gene interactions, and gene-environment interactions; (3) performing experimental tests and interpreting the pathophysiological consequences of risks at the molecular level; and (4) translating these results into direct health benefits.

To identify rare variants in refractive phenotypes, we should use more detailed imputations (e.g., 1000 Genomes) and more in-depth genotyping (e.g., exome sequencing, whole-genome sequencing). Given the high overlap in results, a logical first step will be to perform a meta-analysis of the datasets from the CREAM (Chapter 3.2) and 23andMe[29] studies. This approach will allow us to study low-frequency alleles using the more detailed 1000 Genomes imputations, thus yielding more comprehensive results without the need for *de novo* sequencing. This larger sample size will likely enable us to identify more common genetic variants with relatively modest individual effects. This work is currently ongoing. Moreover, future research should include comprehensive analyses of exome sequencing and whole-genome sequencing experiments, as previous studies showed that approach can be used to identify rare genetic risk factors for myopia[96-98]. In the future, we will focus our analysis on patients with an extreme phenotype (i.e., spherical equivalent of –10 D or worse) and affected families.

Determining the relationship between genes and the environment can also reveal much of the variance in complex traits[92]; therefore, studies of gene-environment interactions are needed as well. In this thesis, we focused primarily on education as an environmental risk factor. Future research should investigate gene-environment interactions using other risk factors for myopia, including near work and outside activity. As misclassification is an important bias appearing in GEWIS[99], future analyses should aim to optimize study designs in order to minimize this effect (e.g. use standardized questionnaires to assess environmental factors). Additionally, studying gene-environment interactions at a molecular level is only possible in animal studies, where environmental factors can be controlled in laboratory settings.

The causative genes may be identified by searching for rare alleles, and they can be validated using functional studies. As discussed above, it might not be necessary to identify the precise causal variant for all identified associations. For functional follow-up studies, we suggest to select those associations identified from our previous GWAS that have a relatively large effect size, associations that were replicated in other studies (e.g., 23andMe[29] and/or follow-up studies), associations that are in or near plausible candidate genes and candidate genes with known expression in the eye.

In addition, knockout animals (for example, genetically modified zebrafish or knockout mice) can be a powerful tool for studying the genetics of myopia at the functional level. The zebrafish embryo develops externally and is transparent; moreover, the embryo's eyes are relatively large and easy to measure, and the emmetropization process develops rapidly during embryogenesis. The genetic control of eye growth and the retinal structure are highly conserved between zebrafish and humans[100]. Several studies have already demonstrated the power of using zebrafish to study myopia genetics and for testing therapeutic interventions, as zebrafish larvae can absorb drugs that are dissolved in the water[101-103]. Mouse models provide another powerful approach for

studying myopia genetics. The mouse genome is highly homologous to the human genome and can be manipulated with relative ease. Genetically modified mice are currently available for a large number of myopia-associated genes. Moreover, previous studies have shown that myopia can be induced in mice[104-106], making mice highly amenable to the study of genetically and environmentally induced myopia[107,108]. Other items that should be considered in future mypia studies are mechanisms governing expression, such as histone modifications, microRNAs, long non-coding RNAs, epigenetics, splice variants, and posttranslational modifications of the encoded proteins.

Systems biology is an additional powerful method for investigating pathways involved in complex genetic diseases. Systems biology is an emerging approach that focuses on complex interactions within biological systems in order to define disease mechanisms based on cell signaling and metabolic networks. Although the cellular basis of myopia pathogenesis is currently unknown, our findings—combined with the findings from 1000 Genomes, exome-sequencing analyses, animal studies, and other published studies—can form the starting point for *in silico* modeling and functional characterization of the pathways that lead to high myopia. Systems biology will likely reveal pathways that are critical for myopia, the cells and/or extracellular compartments involved in these pathways, and putative targets for therapeutic research and prevention modalities[109].

One of the major goals of genomics research is to use GWAS findings to develop clinically relevant gene-based tests and therapeutic strategies targeted to disease-related molecular events[94]. With respect to age-related macular degeneration, considerable progress has been made in these areas[110-112]. When most of the risk factors for a disease are known, these factors can be used to predict the disease, thereby helping future clinical trials select high-risk groups for intervention at a young age. The ultimate goal is to identify high-risk groups according to pathways, and treat these patients with regimens focused on intervention of the major pathways involved. The treatment target is prevention of myopic refractive error from exceeding –6 D.

FINAL REMARKS

Answering the question of why eyes become myopic cannot be answered easily. Many factors determine the pathway from emmetropia to myopia. Although genetic factors provide much of the patient's susceptibility, environmental factors are key players in triggering the conversion to myopia pathogenesis. Many genetic risk variants for myopia remain to be identified, and researchers are exploiting new methods to discover the more rare genetic risk factors, including exome sequencing and whole-genome sequencing. Several studies have shown that lifestyle factors—in particular, education level and outdoor exposure—play a key role in the onset and progression of myopia. Focusing future research on the network of molecules involved, and their response to environmental stimuli, should create new strategies for intervention and prevention, ultimately reducing the prevalence of myopia-related visual impairment and blindness.

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6.2

Summary

SUMMARY

Myopia (nearsightedness) is a highly common eye condition that is predominantly caused by an axial elongation of the eye. Myopia can usually be corrected with negative glasses, contact lenses, and/or laser refractive surgery. Unfortunately, however, high myopia (defined as refractive error greater than –6 diopters) can lead to structural changes in the retina and optic disc, resulting in severe complications such as myopic macular degeneration, glaucoma, and retinal detachment. Although myopia results from an interplay between genetic and environmental risk factors, how these factors interrelate and cause disease at the molecular level remains poorly understood.

The main objectives of this thesis were as follows: 1) assess the current prevalence and visual consequences of myopia and refractive error; 2) identify genetic risk factors for myopia and refractive error; 3) investigate gene-environment interactions; and 4) describe the functional mechanisms that underlie the development of myopia. Our study population included the large population-based Rotterdam Study, the Erasmus Rucphen Family (ERF) Study, the high myopia case control study MYST, many Asian and Caucasian population-based cohort studies from the Consortium for Refractive Error and Myopia (CREAM) and the European Eye Epidemiology (E^3) consortium, and patients from the national RD5000 database.

Chapter 1 gives a general introduction to myopia and refractive error and describes the main aims of this thesis. **Chapter 2** discusses the prevalence and impact of myopia and refractive error. **Chapter 2.1** describes the prevalence of refractive error specifically in Europe. We found that more than half of all adult Europeans have refractive error. The greatest burden from refractive error is associated with myopia, with prevalence rates of 30% and 3% for myopia and high myopia, respectively. The highest prevalence of myopia occurs among young adults, reflecting the rising prevalence of myopia in younger generations. In **Chapter 2.2**, we studied the causes of blindness and low vision in relation to refractive error. We found that visual impairment occurs in one-third of individuals with high myopia, and this impairment is caused primarily by myopic macular degeneration. The highest risk of visual impairment is among individuals with severe refractive error (–10 D or more). We further explored the risks of visual impairment in relation to axial length in **Chapter 2.3**. The risk of visual impairment in high myopes was highly correlated with axial length and reached extreme figures at eyes with an axial length ≥30 mm; >90% of these eyes was visually impaired. These data underscore the need for a proper treatment for high myopia, as reducing progressive eye growth to achieve a lower final axial length would significantly decrease the patient's risk of developing visual impairment later in life. Indeed, each incremental decrease in final AL will improve the patient's long-term prognosis in terms of preserving visual acuity.

In **Chapter 3**, we report on the genetic risk factors that we identified for refractive error and several myopia endophenotypes using genome-wide association studies (GWAS). **Chapter 3.1** and **Chapter 3.2** describe the identification and confirmation of two genetic risk factors for common refractive error. These loci are located on chromosome 15q14 and 15q25, and the closest genes are *GJD2* and *RASGRF1*, respectively. Identifying the functions of these genes has led to new hypotheses regarding the development of myopia, as each gene plays a role in

retinal neurotransmission. In **Chapter 3.3**, we discuss a large-scale GWAS meta-analysis of the CREAM consortium, in which we identified an additional 24 loci associated with refractive error. The associated genes within these loci are involved in neurotransmission, ion transport, retinoic acid metabolism, extracellular matrix remodeling, and eye development. Thus, these pathways fit nicely into the current hypothesis regarding myopia pathogenesis. In **Chapter 3.4**, we report nine loci for axial length that were identified from another GWAS meta-analysis of the CREAM consortium. Two of these genes are involved in Wnt signaling, a pathway that plays a major role in regulating eyeball size. Our meta-analysis of nine myopia and hyperopia genome-wide association studies is presented in **Chapter 3.5**, which provides further evidence that some of the CREAM loci are also associated with these phenotypes. **Chapter 3.6** summarizes the results of a third CREAM meta-analysis in which we identified several novel candidate genes for refractive astigmatism. Furthermore, this work provided evidence to support widespread genetic co-susceptibility to spherical and astigmatic refractive errors.

In **Chapter 4**, we describe the role of education (an environmental risk factor) in the development of myopia, and we provide compelling evidence of a gene-by-environment interaction. In **Chapter 4.1**, we report that the prevalence of myopia is increasing in Europe. We conclude that although education levels have risen and are generally associated with myopia, higher education appears to be an additive—rather than explanatory—factor. In **Chapter 4.2**, we discuss the identification of a significant biological interaction between education and the genetic risk of myopia, represented by our reported associated genetic risk factors. Specifically, subjects with many variants in myopia genes and a high educational level (e.g., university education) were significantly more likely to develop myopia than subjects with only one of these two factors. In **Chapter 4.3**, we discuss the identification of ten novel loci associated with refractive error; these loci were identified in the CREAM consortium using a genome-wide gene-by-environment approach. These data provide convincing evidence that specific genetic variants interact with education to influence refractive development, and they further support a role for GABAergic neurotransmission in the development of myopia.

In **Chapter 5**, we discuss our study of refractive errors in patients with inherited retinal dystrophies. We found that refractive error—and myopia in particular—are common among these patients. Especially patients with congenital stationary night blindness, which is caused by defective retinal signaling from photoreceptors to the ON-bipolar cells, often present with both high myopia and high hyperopia. Patients with X-linked retinitis pigmentosa mainly presented with high myopia. In contrast, patients with cone dystrophies and retinitis pigmentosa generally present with mild myopia. This finding suggests that ON-bipolar cells play a specific role in the development of myopia.

Lastly, **Chapter 6** provides an overview of our main findings, a general interpretation of these findings, and the implications of our results. In addition, we discuss strategies for future research.

The studies described in this thesis have provided considerable insight into the complex genetic and environmental factors that give rise to myopia and refractive error, and they have given us new directions for treating and/or preventing this rising health issue.

6.3

Samenvatting

SAMENVATTING

Myopie (bijziendheid) is een veelvoorkomende oogaandoening die ontstaat door een verlenging van de oogas. Myopie kan doorgaans worden gecorrigeerd met een bril, contactlenzen en/of laserbehandeling of refractiechirurgie. Helaas kan hoge myopie (gedefinieerd als brilsterkte van meer dan -6 dioptrieën) leiden tot structurele veranderingen van het netvlies en de oogzenuw, waardoor ernstige complicaties zoals myope maculadegeneratie, glaucoom en netvliesloslatingen kunnen optreden. Het was allang bekend dat myopie ontstaat door een samenspel tussen genetische factoren en omgevingsfactoren (bijv. veel lezen en weinig buitenspelen), maar het was onbekend om welke genetische factoren het precies ging en hoe de samenhang was tussen deze factoren.

De belangrijkste vragen die we met dit proefschrift wilden beantwoorden waren: 1) Hoe vaak komen myopie en refractieafwijkingen voor en wat zijn de visuele gevolgen ervan? 2) Welke genetische factoren veroorzaken myopie en refractieafwijkingen? 3) Hoe is de samenhang tussen genetische factoren en omgevingsfactoren? en 4) Welke functionele mechanismen liggen ten grondslag aan het ontstaan van myopie?

Onze studiepopulatie bestond uit het Erasmus Rotterdam Gezondheid Onderzoek (ERGO, ook wel Rotterdam Studie genoemd), de Erasmus Rucphen Familie Studie (ERF), de case control MYopie STudie (MYST), Aziatische, Europese, Amerikaanse en Australische studies van het Consortium of Refractive Error and Myopia (CREAM) en het European Eye Epidemiology (E^3) consortium, en patiënten uit de nationale RD5000 database.

Hoofdstuk 1 geeft een algemene introductie over myopie en refractieafwijkingen. **Hoofdstuk 2** geeft weer hoe vaak myopie en refractieafwijkingen voorkomen en wat de visuele gevolgen ervan zijn. In **Hoofdstuk 2.1** onderzochten we hoe vaak refractieafwijkingen voorkomen in Europa. We vonden dat meer dan de helft van alle volwassen Europeanen een refractieafwijking heeft. Myopie komt het vaakst voor; 30% van alle Europeanen is myoop, 3% is hoog myoop. Er is sprake van een stijgende frequentie van myopie bij jongere generaties. In **Hoofdstuk 2.2** hebben we onderzocht hoe vaak mensen met een refractieafwijking blind of slechtziend worden en wat de oorzaken hiervan zijn. We vonden dat een derde van de mensen met een hoge myopie blind of slechtziend wordt, en dat dit voornamelijk veroorzaakt wordt door myope maculadegeneratie. Mensen met een zeer hoge myopie (-10 D of meer) hebben het grootste risico om slechtziend te worden. Daarnaast onderzochten we de relatie tussen oogaslengte en slechtziendheid in **Hoofdstuk 2.3**. Het risico op slechtziendheid wordt hoger naarmate de aslengte langer wordt. Bij een hogere aslengte wordt dit effect duidelijker; ruim 90% van de ogen met een aslengte groter dan 30 mm wordt uiteindelijk slechtziend. Deze data benadrukken de noodzaak van het ontwikkelen van een goede behandeling voor hoge myopie. Zelfs een geringe afname in de uiteindelijke aslengte van het oog van een patiënt met hoge myopie kan de visuele prognose op lange termijn sterk verbeteren.

In **Hoofdstuk 3** doen we verslag van de genetische risicofactoren die we geïdentificeerd hebben voor refractieafwijkingen en myopie met behulp van genoomwijde associatie studies (GWAS). In **Hoofdstuk 3.1** en **Hoofdstuk 3.2** identificeerden en bevestigden we twee genetische risicofactoren voor refractieafwijkingen. Deze genetische factoren liggen op chromosoom 15q14 en 15q25, dichtbij

het *GJD2*- en *RASGRF1*-gen. Beiden genen spelen een rol in het doorgeven van signalen binnen het netvlies en dit leverde de eerste nieuwe inzichten in het ontstaan van myopie op. In **Hoofdstuk 3.3** bespreken we een grootschalige meta-analyse van GWAS studies binnen het CREAM consortium, waarin we nog eens 24 extra genetische factoren voor refractieafwijkingen vonden. De dichtbij gelegen genen zijn betrokken bij doorgeven van signalen en transporteren van moleculen in het netvlies, de vitamine A cyclus, de opbouw van het bindweefsel rondom het oog en bij de ontwikkeling van het oog. Deze functies passen binnen de huidige hypothese over het ontstaan van myopie. In **Hoofdstuk 3.4** vonden wij bij een andere meta-analyse binnen CREAM negen genetische factoren voor aslengte. Twee van deze genen zijn betrokken bij Wnt signalering, dat een belangrijke rol heeft bij het reguleren van de oogbolgrootte. In **Hoofdstuk 3.5** vonden we bewijs dat de genetische factoren voor refractieafwijkingen uit de studies van CREAM ook geassocieerd zijn met de extreme waarden van deze continue factor, namelijk met myopie en hyperopie (verziendheid). In **Hoofdstuk 3.6** rapporteren we over een derde CREAM meta-analyse waarin we nieuwe genetische factoren voor astigmatisme identificeerden.

In **Hoofdstuk 4** beschrijven we de rol van opleidingsniveau (een omgevingsfactor) in de ontwikkeling van myopie, en leveren wij overtuigend bewijs dat er interactie bestaat tussen genetische factoren en omgeving. In **Hoofdstuk 4.1** melden wij dat de frequentie van myopie in Europa toeneemt en dat dit deels verklaard kan worden door een toename in het opleidingsniveau van de bevolking. In **Hoofdstuk 4.2** vinden we interactie tussen opleidingsniveau en genetisch risico op myopie. Personen met een hoog genetisch risico én een hoog opleidingsniveau (bijv. een universitaire studie) hadden een veel grotere kans om myoop te worden dan personen met slechts één van beide factoren. In **Hoofdstuk 4.3** hebben we in CREAM een genoombrede methode gebruikt om gen-omgevingsinteractie op te sporen; op deze manier vonden we tien nieuwe genetische factoren voor refractieafwijkingen die ook interactie vertonen met opleidingsniveau.

In **Hoofdstuk 5** bespreken we onze studie naar refractieafwijkingen bij patiënten met erfelijke retina dystrofieën, aandoeningen van het netvlies. We vonden dat refractieafwijkingen, en myopie in het bijzonder, vaak voorkomen bij deze patiëntengroep. Vooral patiënten met congenitale stationaire nachtblindheid, een aandoening die veroorzaakt wordt door een defect in de signaaloverdracht tussen fotoreceptoren en bipolaire cellen in het netvlies, waren vaak ofwel hoog myoop ofwel hoog hyperoop (verziend). Patiënten met geslachtsgebonden retinitis pigmentosa hadden vaak een hoge myopie. Patiënten met kegeldystrofie en retinitis pigmentosa daarentegen hadden over het algemeen een milde myopie. Deze bevinding suggereert dat bipolaire cellen een belangrijke rol spelen bij het ontstaan van myopie.

Tenslotte geeft **Hoofdstuk 6** een overzicht van onze belangrijkste bevindingen, een algemene interpretatie en de betekenis van onze resultaten. Daarnaast bespreken we strategieën voor toekomstig myopieonderzoek.

De in dit proefschrift beschreven studies hebben veel inzicht verschaft in de complexe achtergrond van myopie en refractieafwijkingen, die ontstaan door een samenspel tussen genetische factoren en omgevingsfactoren. Deze geven ons nieuwe richtingen voor onderzoek naar mogelijkheden tot het behandelen en/of voorkomen van dit toenemende gezondheidsprobleem.

PUBLICATIONS AND MANUSCRIPTS ON WHICH THIS THESIS IS BASED

1. Soulouki, A.M., **Verhoeven, V.J.** (shared 1st author) *et al.* A genome-wide association study identifies a susceptibility locus for refractive errors and myopia at 15q14. *Nat Genet* **42**, 897-901 (2010). [Chapter 3.1]

2. **Verhoeven, V.J.** *et al.* Large scale international replication and meta-analysis study confirms association of the 15q14 locus with myopia. The CREAM consortium. *Hum Genet* **131**, 1467-80 (2012). [Chapter 3.2]

3. Cheng, C.Y., **Verhoeven, V.J.** (2nd author) *et al.* Nine loci for ocular axial length identified through genome-wide association studies, including shared loci with refractive error. *Am J Hum Genet* **93**, 264-77 (2013). [Chapter 3.5]

4. **Verhoeven, V.J.** *et al.* Education influences the role of genetics in myopia. *Eur J Epidemiol* **28**, 973-80 (2013). [Chapter 4.2]

5. **Verhoeven, V.J.** *et al.* Genome-wide meta-analyses of multiancestry cohorts identify multiple new susceptibility loci for refractive error and myopia. *Nat Genet* **45**, 314-8 (2013). [Chapter 3.3]

6. Klaver C.C., **Verhoeven, V.J.** (last author) *et al.* Why do Eyes Become Myopic? *CRST Europe* (2014). [Chapter 1.2]

7. Simpson, C.L., **Verhoeven, V.J.** (5th author) *et al.* Genome-wide meta-analysis of myopia and hyperopia provides evidence for replication of 11 loci. *PLoS One* **9**, e107110 (2014). [Chapter 3.4]

8. Li, Q., **Verhoeven, V.J.** (5th author) *et al.* Genome-wide association study for refractive astigmatism reveals genetic co-determination with spherical equivalent refractive error: the CREAM consortium. *Hum Genet* **134**, 131-46 (2015). [Chapter 3.6]

9. **Verhoeven, V.J.** *et al.* Visual consequences of refractive errors in the general population. *Ophthalmology* **122**, 101-9 (2015). [Chapter 2.2]

10. Williams, K.M, **Verhoeven, V.J.** (2nd author) *et al.* Prevalence of refractive error in Europe: the European Epidemiology Eye (E^3) Consortium. *Eur J Epidemiol* **30**. 305-15 (2015). [Chapter 2.1]

11. Williams, K.M, **Verhoeven, V.J.** (2nd author) *et al.* Increasing prevalence of myopia in Europe and the impact of education. Accepted for publication in *Ophthalmology (2015).* [Chapter 4.1]

12. Fan, Q., **Verhoeven, V.J.** (shared first author) *et al.* Genome-wide joint meta-analyses of SNP and interaction with education identify novel loci for refractive error in CREAM. S*ubmitted*. [Chapter 4.3]

13. **Verhoeven, V.J.** *et al.* Axial length and visual function in high myopia*. Submitted.* [Chapter 2.3]

14. Hendriks, M., **Verhoeven, V.J.** (shared first author) *et al.* Development of refractive errors: what can we learn from inherited retinal dystrophies? *Submitted.* [Chapter 5.1]

OTHER PUBLICATIONS

15. van Eerde, A.M., **Verhoeven V.J.** (shared 1st author) *et al.* Is joint hypermobility associated with vesico-ureteral reflux? An assessment of 50 patients. *BJU Int* **109**, 1243-8 (2012).

16. Hoehn, R., **Verhoeven, V.J.** (3rd author) *et al.* Population-based meta-analysis in Caucasians confirms association with COL5A1 and ZNF469 but not COL8A2 with central corneal thickness. *Hum Genet* **131**, 1783-93 (2012).

17. Lopes, M.C., **Verhoeven, V.J.** (3rd author) *et al.* Identification of a candidate gene for astigmatism. *Invest Ophthalmol Vis Sci* **54**, 1260-7 (2013).

18. Lu, Y. , **Verhoeven, V.J**. (co-author) *et al.* Genome-wide association analyses identify multiple loci associated with central corneal thickness and keratoconus. *Nat Genet* **45**, 155-63 (2013).

19. Stambolian, D., **Verhoeven, V.J.** (co-author) *et al.* Meta-analysis of genome-wide association studies in five cohorts reveals common variants in RBFOX1, a regulator of tissue-specific splicing, associated with refractive error. *Hum Mol Genet* **22**, 2754-64 (2013).

20. Springelkamp, H. **Verhoeven, V.J.** (co-author) *et al.* Meta-analysis of genome-wide association studies identifies novel loci that influence cupping and the glaucomatous process. *Nat Commun* **5**, 4883 (2014).

21. Ohno-Matsui, K., **Verhoeven, V.J.** (co-author) *et al.* International Photographic Classification and Grading System for Myopic Maculopathy. *Am J Ophthalmol* (2015).

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Daarnaast gaat mijn dank uit naar prof. dr. Cornelia van Duijn, dr. Anneke Kievit, prof. dr. Nomdo Jansonius, prof. dr. Christopher Hammond en prof. dr. Frank Schaeffel, leden van de grote commissie. Cock, wat was ik blij met jou toen we gescoopt dreigden te worden door 23andMe en Caroline op de Kilimanjaro stond! Dankzij jouw kordate optreden kwam uiteindelijk toch nog alles goed. Die aanpak heb ik sowieso erg gewaardeerd gedurende mijn gehele promotietraject. Anneke, jij stelde me voor aan Caroline en hebt me daarmee in het zadel geholpen naar dit mooie onderzoeksproject. Beste Nomdo, ik wil graag nog een keer mee wadlopen! Dear Chris, we have been working together from the very first day of my PhD project, it is great to have you on board! My thesis defense committee would not have been complete without you. Dear Frank, I have admired your work since I attended the IMC conference in Tübingen. It is a great honour that you are joining my thesis defense.

Lieve oog-epi collega's, wat heb ik dankzij jullie een fijne tijd gehad! Ada & Corina, zonder jullie is het ERGO (en MYST!) oog onderzoek nergens! Superveel dank voor alle hulp en gezelligheid de afgelopen jaren. Magda, sinds jij er bent loopt alles soepeler en stroomt het subsidiegeld binnen.

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My PhD project would not have been successful without the great collaborations with CREAM and E^3. I could fill a whole chapter with names of collaborators and co-authors from these consortia: a HUGE THANKS to all of you. I would like to acknowledge the great efforts of Dwight Stambolian. A special mention for my CREAM WG1 buddies Rob and Pirro, we are a great team! Dear Claire, Ching-Yu, Qing, Jez, Joan, and Katie, all your hard work lead to very successful CREAM and E^3 papers, and hopefully many more to come. Anthony, thanks for coming up with the catchy title of my thesis. It is always good to have you around during conferences.

A special thanks to prof. Tien Wong, prof. Ching-Yu Cheng, and prof. Seang Mei Saw for giving me an amazing time in Singapore. Qiao, Jiemin & Queenie, you are the best! Dear Mojo, Wiet & Mark, Juul & Saurubh, Myrte & Eef, thanks for being my Singapore family.

Zonder de vele studiedeelnemers was dit proefschrift er überhaupt niet geweest. Dank voor jullie essentiële bijdrage aan alle oogonderzoeken!

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Wokkels en aanhangsels, wat is het altijd een feestje bij Sem & Lied in Limmen! Er zijn heel veel vooroordelen over schoonfamilies, maar niets daarvan is waar. De laatste maanden gebruikte ik Limmen zo af en toe als een toevluchtsoord waar ik rustig kon werken en waar het mij aan niets ontbrak. San, Fem, Mars en Daan, de jaarlijkse weekendjes zijn fantastisch en ik hoop nog veel Europese steden met elkaar te mogen bezoeken.

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Erasmus MC
Universitair Medisch Centrum Rotterdam

PHD PORTFOLIO

SUMMARY OF PHD TRAINING AND TEACHING

Name PhD student: Virginie Verhoeven
Erasmus MC Department: Ophthalmology/Epidemiology
Research School: NIHES
PhD period: 2009-2015
Promotors: prof. dr. C.C.W. Klaver and prof. dr. J.R. Vingerling

1. PhD training	Year	Workload (ECTS*)
Courses		
Master of Health Sciences, Genetic Epidemiology (NIHES)	2009-2012	70
Workshop on Photoshop and Illustrator CS5 (MolMed)	2011	0.3
Workshop on InDesign CS5 (MolMed)	2011	0.2
Biomedical English Writing and Communication (David Alexander)	2011	4.0
Next-Generation Sequencing course (MGC, Boerhaave)	2011	0.9
Seminars, symposia and workshops		
Myopia workshop for opticians and optometrists, Rotterdam (oral presentation)	2009	1.0
Myopia workshop for ophthalmologists, Rotterdam (oral presentation)	2009	1.0
LVAO course optics and refractive surgery, Utrecht	2010	0.3
International Course Genetics in Retinal Disease, Rotterdam	2010	0.3
Research Day Rotterdam Eye Hospital, Rotterdam (oral presentation)	2010	1.0
ARVO-NED, Utrecht (oral presentation)	2010	1.0
1st European Eye Epidemiology Workshop, Bordeaux, France (oral presentation)	2011	1.0
CREAM consortium meeting 2012, Rotterdam (oral presentation)	2012	1.0
CREAM consortium meeting 2012, Sardinia, Italy (oral presentation)	2012	2.0
3rd European Eye Epidemiology Workshop, Bordeaux, France	2013	0.3
CREAM consortium meeting 2013, Singapore (oral presentation)	2013	2.0
CREAM consortium meeting 2014, Hong Kong (oral presentation)	2014	2.0
Co-organizer of the Epi 2020 meetings, department of Epidemiology, Erasmus MC	2012-2013	1.0
Research seminars, department of Epidemiology, Erasmus MC	2009-2013	4.5
National conferences		
NOG Annual Meeting 2010, Maastricht	2010	0.3
SEOHS 2010, Rotterdam (oral presentation)	2010	1.0
15th Molecular Medicine Day, Rotterdam (invited oral presentation)	2011	1.0
NOG Annual Meeting 2011, Maastricht (oral presentation)	2011	1.0
NOG Annual Meeting 2012, Groningen (oral presentation)	2012	1.0
NOG Annual Meeting 2013, Groningen (oral presentation)	2013	1.0
2nd Dutch Ophthalmology PhD Day, Nijmegen (oral presentation)	2013	1.0

International conferences		
ARVO Annual Meeting 2010, Fort Lauderdale, USA (oral presentation)	2010	1.0
International Myopia Conference 2010, Tübingen, Germany (invited oral presentation)	2010	1.0
ARVO Annual Meeting 2011, Fort Lauderdale, USA (poster presentation)	2011	1.0
ARVO Annual Meeting 2012, Fort Lauderdale, USA (oral presentation)	2012	1.0
ARVO Annual Meeting 2013, Seattle, USA (oral presentation)	2013	1.0
ARVO Annual Meeting 2014, Orlando, USA (oral presentation)	2014	1.0
APGC-ISOHK 2014, Hong Kong (invited oral presentation)	2014	1.0
ASHG Annual Meeting 2014, San Diego, USA (poster presentation)	2014	1.0
ARVO Annual Meeting 2015, Denver, USA (invited oral presentation)	2015	1.0
2. Teaching - Supervising Master's theses		
Supervising research internship, King Wong (20 weeks)	2011	1.4
Supervising research internship, Michelle Hendriks (20 weeks)	2013	1.4
Supervising research internship, Martine Snabel (20 weeks)	2013	1.4
3. Other		
Organizing committee Myopia workshops for opticians, optometrists and ophthalmologists, Rotterdam	2011	2.0
Founder and co-organizer of the 1st Dutch Ophthalmology PhD Day, Nijmegen	2012	3.0
Chair of myopia session, ARVO Annual Meeting 2014, Orlando, USA	2014	0.1
Secretary of the CREAM consortium	2010-today	4.0
Reviewer for several international journals	2009-today	1.0
Organizing committee CREAM consortium meetings Rotterdam, Sardinia, Singapore and Hong Kong	2012-today	4.0

* 1 ECTS (European Credit Transfer System) equals a workload of 28 hours.

ABOUT THE AUTHOR

Virginie Verhoeven was born on March 25, 1984 in Nieuwegein, the Netherlands from a myopic mother and a hyperopic father. In 2002, she graduated cum laude from secondary school 'Erasmiaans Gymnasium' in Rotterdam, which is just across the street from Erasmus Medical Center. During medical school at the University of Utrecht, she encountered the interesting aspects of clinical genetics, and she spent several internships at the department of Medical Genetics of the University Medical Center Utrecht. After obtaining her medical degree in 2008, she started to work as a resident at the department of Clinical Genetics of Erasmus Medical Center in Rotterdam. She moved to the departments of Ophthalmology and Epidemiology in 2009 to start the work described in this thesis under supervision of prof. dr. C.C.W. Klaver and prof. dr. J.R. Vingerling. In 2012, she obtained a Master of Health Sciences in Genetic Epidemiology at the Netherlands Institute for Health Sciences (NIHES). In 2013, she spent 6 months of her PhD project working on gene-environment interactions at the Singapore Eye Research Institute and the National University of Singapore under supervision of prof. dr. Tien Y. Wong. Virginie received several prizes for her thesis work including the MolMed Postgraduate School Publication Award (2011), the CHARGE Consortium Early Career Award (2013), the Bayer Ophthalmology Research Award (BORA, 2013), and the Ludwig von Sallmann Clinician-Scientist Award (2015). In 2014, she returned to the department of Clinical Genetics at Erasmus Medical Center in Rotterdam where she currently works as a resident under supervision of dr. J.A. Kievit.

Stellingen behorende bij het proefschrift

WHAT CAUSES MYOPIA?
Complex genetics and epidemiology of a common condition

1. 1 op de 3 personen met hoge bijziendheid (meer dan -6 dpt) wordt blind of slechtziend. *(dit proefschrift)*

2. Het GJD2-gen op chromosoom 15q14 speelt een belangrijke rol bij het ontstaan van myopie. *(dit proefschrift)*

3. Neurotransmissie, extracellulaire matrix remodellering, retinolzuurmetabolisme en oogontwikkeling zijn belangrijke pathways voor het ontstaan van myopie. *(dit proefschrift)*

4. Personen met een hoog opleidingsniveau én een hoog genetisch risico hebben een vele malen grotere kans op bijziendheid dan personen met slechts één van deze factoren. *(dit proefschrift)*

5. Bipolaire cellen lijken een sleutelfunctie te vervullen bij het ontstaan van hoge myopie bij patiënten met een retinadystrofie. *(dit proefschrift)*

6. Het anamnestisch verkrijgen van brilsterkte data is net zo goed als het gedetailleerd meten van brilsterktes voor genetische studies naar myopie. *(R. Wojciechowski & P.G. Hysi, PLoS Genet, 2013 Apr;9(4):e1003442).*

7. Frequent (daily) consumption of curry may be protective against high myopia in Singaporean adults of Indian ethnicity. *(A. Anuar, ARVO 2013, Invest Ophthalmol Vis Sci 2013;54:5705)*

8. Als klinisch genetici zich naast Mendeliaanse ziekten ook gaan focussen op complex genetische aandoeningen, zal dit een deel van de werkgelegenheids problematiek in ons vakgebied oplossen.

9. Dat bijziendheid geassocieerd is met een hoger opleidingsniveau betekent niet dat verzienden dom zijn.

10. Liever bijziend dan kortzichtig.

11. The best journey's in life are those that answer questions you never thought to ask. *(Rich Ridgeway)*

Virginie J. M. Verhoeven, Rotterdam, 16 juni 2015